REMARKS

Claims 1, 5, 6, 9-11 and 14-26 are pending. Claims 2-4, 7, 8, 12 and 13 are canceled; claims 1, 5, 6, 9, 10, 16 and 17 are amended; and claims 20-26 are added herein.

Information Disclosure Statements with Forms PTO-1449 were filed in the above-identified patent application on March 26, 2001 and August 29, 2001. Applicant has not yet received back from the Examiner copies of the Forms PTO-1449 initialed to acknowledge the fact that the Examiner has considered the cited disclosed information. Thus, the Examiner is respectfully requested to initial and return to the undersigned copies of the subject Forms PTO-1449. For the convenience of the Examiner, copies of the forms are attached.

A Request for Approval of Drawing Corrections was also filed on August 29, 2001. Applicant respectfully requests that the Examiner approve the proposed drawing corrections. It is noted that an explanation of the proposed drawing corrections is included at page 3 of the Preliminary Amendment filed August 29, 2001.

Claims 3, 7, 8 and 13 are withdrawn from consideration. Claims 3, 7, 8 and 13 are canceled herein. The specification is objected to for lacking an Abstract. An Abstract is added herein.

Claims 6 and 16 are objected to for referring to a figure. Claims 6 and 16 have been amended to refer to SEQ ID NO: 7 rather than Fig. 2. In addition, claim 4 is objected to based on nonagreement of tense. Claim 4 has been canceled rendering the rejection moot. Thus, the objections should be withdrawn.

Claims 1, 2, 4-6, 9-12 and 14-19 are rejected to under 35 U.S.C. §112, second paragraph, based on the recitation of the term "complementary." Claims 1, 9, 10 and 17 have been amended by replacing the term "complementary" with the term "hybridizes." Support for the amendment appears in the specification at page 5, line 9. The term "hybridizes" is not indefinite because the skilled man of the art would know that the measure of hybridization

required is in order to interfere in a sequence specific manner with the process of mRNA translation into protein, as taught by the specification on page 4, lines 18-20. Such interference of mRNA translation may be determined by standard methods well known to the skilled artisan, and do not require undue experimentation. Therefore, the rejection under 35 U.S.C. §112, second paragraph, should be reconsidered and withdrawn.

Claims 1, 2, 4-6, 10-12 and 14-19 are rejected under 35 U.S.C. §112, first paragraph, for allegedly being not enabled. Applicant respectfully traverses the rejection.

The Office Action at page 6 indicates that "the therapeutic use of antisense oligonucleotides was a highly unpredictable art due to obstacles that continue to hinder the therapeutic application of antisense *in vivo*." It is respectfully submitted that this is not accurate, as indicated in the attached Declaration under Rule 132. On the contrary, it is respectfully submitted that one of ordinary skill in the art could practice the present invention without undue experimentation.

In particular, the present application describes the expression of cDNA in an antisense orientation within an expression vector. It is respectfully submitted that utilizing antisense technology in this way is enabled, as demonstrated by the four references discussed in and attached to the attached Declaration, which describe the successful use of antisense therapies. The references cited by the Examiner, particularly to the extent that they are directed to antisense ODNs (oligodeoxynucleotides), do not teach or suggest that the techniques described in the present application cannot be successfully be used and that these techniques cannot provide target accessibility, or that the techniques provides unpredictable nonantisense effects. Quite the contrary, the attached references specifically teach that the methodology described in the present application can effectively be used. Thus, the Patent Office has provided no basis to assume that undue experimentation would be required to practice the present invention. Instead, as expressed in the Declaration under Rule 132, one of ordinary skill of the art is of the opinion that undue experimentation would not be required.

Claims 1, 2, 4-6, 10-12 and 14-19 are enabled by the present specification. Therefore, the rejection under 35 U.S.C. §112, first paragraph, should be reconsidered and withdrawn.

Claim 9 is rejected under 35 U.S.C. §102 over Schaeffer et al., Chaikof et al., Mattson et al., and Herbert et al. Applicant respectfully traverses the rejection.

Schaeffer, Chaikof, Mattson and Herbert each teach antisense oligodeoxynucleotides (ODNs) having 15-24 bases. These references do not teach an expression vector comprising an antisense molecule comprising a nucleotide sequence which hybridizes to an RNA sequence of a thrombin receptor protein, wherein the nucleotide sequence consist of between 250 and 600 base pairs. Therefore, the rejection of claim 9 over these references should be reconsidered and withdrawn.

Claim 9 is rejected under 35 U.S.C. §102(b) over Even-Ram et al. However, Even-Ram is not prior art under 35 U.S.C. §102(b). In particular, this reference was published in August of 1998, which is less than a year before the February 5, 1999 international filing date.

Moreover, it is noted that the present inventor is one of the authors of Even-Ram. In fact, she is the principal author, the other authors being her students. As indicated in the Declaration under 37 C.F.R §1.132, to the extent that the subject matter recited in the rejected claims is also disclosed in Even-Ram, the present inventor is the only inventor of this subject matter. Thus, Even-Ram is also not prior art 37 C.F.R. §102(a).

Even-Ram is clearly not prior art under 35 U.S.C. §102(b). Therefore, the rejection in view of this section should clearly be reconsidered and withdrawn. In addition, for the reasons discussed above, Even-Ram should not be applied under a new paragraph of §102.

In view of the foregoing, it is respectfully submitted that this application is in condition for allowance. Favorable reconsideration and prompt allowance of claims 1, 5, 6, 9-11 and 14-26 are earnestly solicited.

Should the Examiner believe that anything further would be desirable in order to place this application in even better condition for allowance, the Examiner is invited to contact the undersigned at the telephone number set forth below.

Respectfully submitted,

James A. Oliff
Registration No. 27,075

Melanie L. Mealy
Registration No. 40,085

JAO:MLM/jam

Attachments:
- Abstract
- Forms PTO-1449 (2)
- Declaration under Rule 132 w/Annexes A-E
- Declaration under 37 C.F.R. §1.132

Date: June 23, 2003

OLIFF & BERRIDGE, PLC
P.O. Box 19928
Alexandria, Virginia 22320
Telephone: (703) 836-6400

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of

Rachel BAR-SHAVIT

Application No.: 09/744,679

Filed: April 11, 2001

For: METHOD FOR TREATMENT OF INVASIVE CELLS

Group Art Unit: 1635

Examiner: K. Lacourciere

Docket No.: 108366

DECLARATION UNDER 37 C.F.R. §1.132

I, Rachel Bar-Shavit, hereby declare:

1. That I am the named and true inventor in the above-captioned patent application and that I am the inventor of the subject matter defined by rejected claims 1-19 in said patent application and that I am the only inventor thereof;

Rachel Bar-Shavit

2. That I am a ~~co~~ principal author, along with Sharona Even-Ram, Beatrice Uziely, Patrizia Cohen, Sorina Grisaru-Granovsky, Miriam Maoz, Yoav Ginzburg, Reuven Reich and Israel Vlodavsky, of the article entitled "Thrombin receptor overexpression in malignant and physiological invasion processes," Nature Medicine, Vol. 4, No. 8, pp. 909-914 (August 1998); and

3. That to the extent that the subject matter recited in the rejected claims of the above-captioned patent application is also disclosed in said publication, I am the inventor of said subject matter disclosed in said publication and am the only inventor thereof.

4. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and/or imprisonment under Section 1001 of Title 18

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of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Date: 22.6.03

Rachel Bar-Shavit

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of BAR-SHAVIT, RACHEL

Serial No. 09/744,679 Group Art Unit: 1635

Filed: 04/11/2001 Examiner: Lacourciere, K.A.

For: **METHOD FOR TREATMENT OF INVASIVE CELLS**

DECLARATION
under Rule 132

Commissioner of Patents and Trademarks
Washington, D.C. 20231

I, Rachel Bar Shavit, an Israeli citizen residing at Ramat Sharet, Jerusalem, Israel, hereby declare:

1. I am currently a Research Group Team Leader (Senior Lecturer) in the Departments of Experimental Medicine & Cancer Research and of Oncology, Hadassah-Hebrew University Hospital, Jerusalem, Israel.

2. My *Curriculum Vitae* and list of publications is attached herewith as Annex "A". My fields of expertise include biology of tumors and angiogenesis.

3. I am the inventor of U.S. Patent Application No. 09/744,679 (hereinafter *"the application"*). The application describes the use of an antisense molecule comprising a nucleotide sequence which is complementary to an RNA sequence of a PAR protein for treating metastatic tumor cells (hereinafter *"the invention"*).

4. I am also familiar with the comments of the examiner in the office action dated December 23, 2002 (hereinafter *"the office action"*).

5. It is stated on page 6 of the office action that "*the therapeutic use of antisense oligonucleotides was a highly unpredictable art due to obstacles that continue to hinder the therapeutic application of antisense* in vivo". In my professional opinion, this statement is not accurate.

6. Firstly, I would like to point out that the invention relates to the expression of cDNA in an antisense orientation within an expression vector (see Example 3 on page 15 of the application), and not to antisense oligonucleotides (ODN) which are generally <30 base pairs in length. Therefore, the alleged unpredictability of the use

Considered KAL 08.27.03

of ODNs is not relevant to the invention.

7. Secondly, I believe that antisense therapeutics has matured and can be considered as an acceptable method of treatment not requiring undue experimentation. In support of the above, I wish to mention the following examples of the successful use of antisense therapeutics, particularly in the treatment of metastatic tumor cells:

One. Jen & Gewirtz (Stem Cells (2000) 18:307-319) present a thorough review of the subject, including the introduction of antisense nucleic acids into a cell in the form of RNA expressed from a vector which has been transfected into the cell (Annex B);

Two. Yang, et al (J. Gastroent. Heptol. (2003) 18:296-301) describe cells transfected with a plasmid vector expressing IGF-IIR cDNA in the antisense orientation. The antisense genes significantly restrained the malignant behavior of human hepatoma cells (Annex C);

Three. Tavian, et al (Cancer Gene Ther. (2003) 10:112-20) describe a cell line stably transfected with an expression vector containing a u-PA cDNA in the antisense orientation. The transfected cells showed a significant reduction in proliferation, Matrigel invasion and motility assays (Annex D);

Four. Zhang, et al (Oncogene (2003) 22:2405-16) describes suppression of the malignant phenotype by the expression of the full-length antisense human telomerase RNA delivered by an adenovirus vector (Annex E).

8. It is further stated on page 7 of the office action that "*cell culture examples are generally not predictive of* in vivo *inhibition*". Here also, I disagree.

9. Almost all progress in biomedical research begins with experimental work using tissue culture and other *in vitro* methods. This approach is universally accepted in the scientific world as a first step towards developing *in vivo* treatments. If, as the examiner claims, cell culture examples were not predictive of *in vivo* applications, their use would not be as widespread as it is. In fact, all of the references cited by the Examiner against Claim 9 base their results on cell culture examples. Schaeffer uses human umbilical vein endothelial cells, Chaikof and Herbert use smooth muscle cells

and Mattson uses a human neuroblastoma cell line. It thus appears that the Examiner himself accepts these references as serious scientific papers, including the methods used by these authors.

10. The undersigned declares further that all statements made herein of her own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: June 16/ 03

Rachel Bar Shavit

Dr. Rachel Bar Shavit

ANNEX A

CURRICULUM VITAE

Name: Rachel Bar-Shavit, Ph.D.
Marital Status: Married, two children

Education:

1971-1974	B.Sc., Cum laude, Bar-Ilan University, Ramat-Gan, Israel.
1974-1977	M.Sc. Life Sciences, The Feinberg Graduate School, Weizmann Institute of Science, Rehovot, Israel. Instructor: Prof. Yosef Aloni, Department of Genetics Thesis: "Regulation of Transcription in Polyoma Virus"
1978-1982	Ph.D., Life Sciences, Bar-Ilan University. Instructor: Dr. Asher Shainberg Thesis: "Studies on Acetyl Choline Receptor Synthesis and Characterization In Vitro"

Professional Experience:

1996- present	Senior Lecturer, Department of Experimental Medicine & Cancer Research and Department of Oncology, Hadassah-Hebrew University Hospital, Jerusalem.
2001	Sabbatical at Harvard University, Dept. of cell Biology
1990-1995	Lecturer, Department of Experimental Medicine & Cancer Research and Department of Oncology, Hadassah-Hebrew University Hospital, Jerusalem.
1987-1989	Research Associate in Oncology, Department Oncology, Hadassah - Hebrew University Hospital, Sharett Institute, Jerusalem.
1985-1986	Research Associate in Pathology, Department of Pathology, Washington University School of Medicine and The Jewish Hospital of St. Louis, St. Louis, Missouri.

1982-1985	Postdoctoral Fellow, Department of Pathology, Washington University School of Medicine and The Jewish Hospital of St. Louis, St. Louis, Missouri. Instructor: Dr. George D. Wilner Research Topic: "Thrombin interaction with monocyte/macrophages."

Rachel Bar-Shavit, Ph.D.

Membership: The American Society for Cell Biology

Israel Society of Hematology

American Heart Association

Honors:

1983: Postdoctoral Fellowship Award, American Heart Association

1990: The Hebrew University, Faculty Prize in memory of Prof. Bagriel Issac- Distinguished Res

1992: The Sanofi Foundation for Thrombosis Research, Scholarship for a Young Academic Resea in Hemostasis, Inflammation and Immunology

1994: The Hebrew University, Faculty Prize for Distinguished Research Project

1999: "The Israel Cancer Association"- the 50th Anniversary Grant Award

2001: " The George and Eva Klein" price, for distinguished research- proposal in tumor biology Israel Scientific Fund (Academia).

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ANNEX B

[redacted]

Suppression of Gene Expression by Targeted Disruption of Messenger RNA: Available Options and Current Strategies

KUANG-YU JEN,[a] ALAN M. GEWIRTZ[b]

[a]Department of Cell and Molecular Biology, University of Pennsylvania School of Medicine, the [b]Department of Medicine and the Cancer Center, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA

Key Words. *RNA · DNA oligonucleotide · Hammerhead ribozymes · Hairpin ribozymes · Gene targeting · Targeted gene disruption · Antisense*

ABSTRACT

At least three different approaches may be used for gene targeting including: A) gene knockout by homologous recombination; B) employment of synthetic oligonucleotides capable of hybridizing with DNA or RNA, and C) use of polyamides and other natural DNA-bonding molecules called lexitropsins.

Targeting mRNA is attractive because mRNA is more accessible than the corresponding gene. Three basic strategies have emerged for this purpose, the most familiar being to introduce antisense nucleic acids into a cell in the hopes that they will form Watson-Crick base pairs with the targeted gene's mRNA. Duplexed mRNA cannot be translated, and almost certainly initiates processes which lead to its destruction. The antisense nucleic acid can take the form of RNA expressed from a vector which has been transfected into the cell, or take the form of a DNA or RNA oligonucleotide which can be introduced into cells through a variety of means. DNA and RNA oligonucleotides can be modified for stability as well as engineered to contain inherent cleaving activity.

It has also been proven that because RNA and DNA are very similar chemical compounds, DNA molecules with enzymatic activity could also be developed. This assumption proved correct and led to the development of a "general-purpose" RNA-cleaving DNA enzyme. The attraction of DNAzymes over ribozymes is that they are very inexpensive to make and that because they are composed of DNA and not RNA, they are inherently more stable than ribozymes.

Although mRNA targeting is impeccable in theory, many additional considerations must be taken into account in applying these strategies in living cells including mRNA site selection, drug delivery and intracellular localization of the antisense agent. Nevertheless, the ongoing revolution in cell and molecular biology, combined with advances in the emerging disciplines of genomics and informatics, has made the concept of nontoxic, cancer-specific therapies more viable then ever and continues to drive interest in this field. *Stem Cells 2000;18:307-319*

INTRODUCTION

The notion that gene expression could be modified through use of exogenous nucleic acids derives from studies by *Paterson et al.* who first used single-stranded DNA to inhibit translation of a complementary RNA in a cell-free system in 1977 [1]. One year later, *Zamecnik* and *Stephenson* noted that a short (13nt) DNA oligonucleotide reverse complementary in sequence (antisense) to the Rous sarcoma virus could inhibit viral replication in culture [2]. This observation is credited as being among the first to suggest the therapeutic utility of antisense nucleic acids, a concept which ultimately led to the awarding of a Lasker Prize in Medicine to *Dr. Zamecnik*. In the mid 1980s, the existence of naturally occurring antisense RNAs and their role in regulating gene expression was demonstrated [3-5]. These observations were particularly important because the

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fact that naturally occurring antisense nucleic acids played a role in regulating gene expression lent support to the belief that exogenously introduced reverse complementary nucleic acids might be utilized to manipulate gene expression in living cells. These seminal papers, and the literally thousands which have followed, have stimulated the development of technologies employing nucleic acids to manipulate gene expression. Virtually all available methods rely on some type of nucleotide sequence recognition for targeting specificity, but differ where and how they perturb the flow of genetic information [6]. Simply stated, strategies for modulating gene expression may be thought of as being targeted to the gene itself, or to the gene's messenger RNA (mRNA). Since this review will be focused on strategies aimed at disrupting the use of mRNA, antigene strategies will be addressed only briefly and mainly for the sake of completeness.

Antigene Strategies

At least three different approaches may be utilized for direct gene targeting. The "gold standard" is the gene "knock-out" achieved by homologous recombination [7, 8]. This approach results in the actual physical disruption of the targeted gene as a result of crossover events which occur during cell division between the targeting vector and the gene selected for destruction (Fig. 1A). Homologous recombination is extremely powerful, but the technique is hampered by the fact that it remains inherently inefficient, time-consuming, and expensive. While improvement in the efficiency of this process has been achieved [9, 10], this is a method which remains restricted to use in cell lines and animal models, if for no other reason than selection is required to find the cells in which the desired events have taken place. In clinical situations where high efficiency gene disruptions are required, it seems unlikely that this approach will serve as a useful therapeutic modality anytime in the foreseeable future.

A second option for gene targeting employs synthetic oligodeoxynucleotides (ODN) capable of hybridizing with double-stranded DNA [11-13]. Such hybrids are typically formed within the major groove of the helix, though hybridization within the minor groove has also been reported [14]. In either case, a triple-stranded molecule is produced, hence the origin of the term triple helix-forming oligodeoxynucleotide (TFO) (Fig. 1B). TFOs do not destroy a gene but prevent its transcription either by preventing unwinding of the duplex or preventing binding of transcription factors to the gene's promoter. TFO sequence requirements are based on the need for each base comprising the TFO to form two hydrogen bonds (Hoogsteen bonds) with its complementary base in the duplex. This constrains TFOs to hybridization with the purine bases composing polypurine-polypyrimidine tracks within the DNA. The targeting efficiency of TFOs is further constrained by a number of factors, including need for divalent cations, and perhaps most importantly, by access to DNA compacted within the chromosome structure. Recent experiments from *Wang et al.* and *Kochetkova et al.* have provided evidence that triple helix formation can occur in living cells, suggesting that these difficulties may ultimately be overcome [15-17]. If shown practical, it has also been postulated that TFOs may prove useful in the treatment of certain genetic disorders such as sickle cell anemia, and hemophilia B, where their ability to trigger repair mechanisms might be used to correct single base pair mutations responsible for the disease [15, 18-20].

Final approaches worth mentioning are the use of specific nucleic acid sequences to act as "decoys" for transcription factors [21, 22], and the use of polyamides and

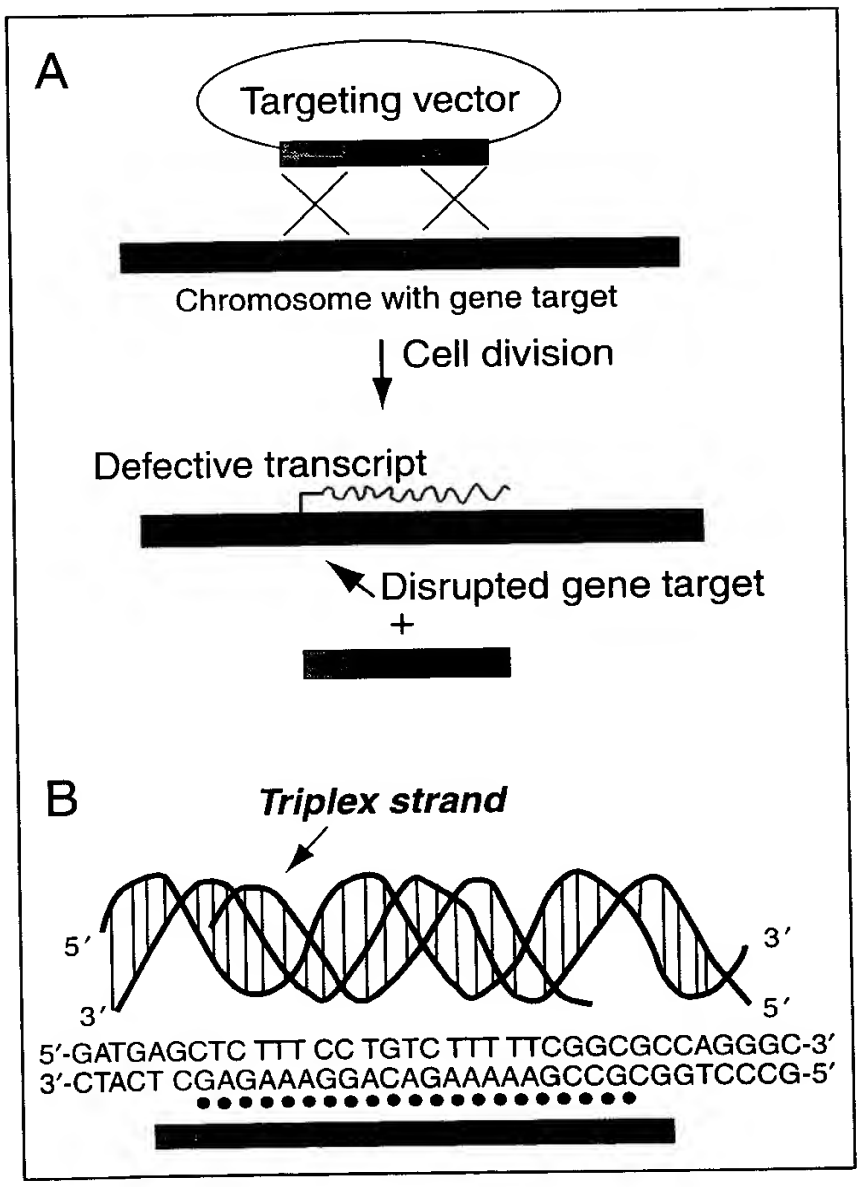

Figure 1. A) Targeting vector; B) Triplex strand. *Adapted from [6].*

other natural DNA-binding molecules called lexitropsins, that bind to specific bases in the minor groove of DNA [23, 24]. The use of decoy molecules evolves from the knowledge that transcription factor proteins recognize and bind specific DNA sequences. In theory then, it is possible to synthesize nucleic acids which will effectively compete with the native DNA sequences for available transcription factor proteins in vivo. If effective, the rate of transcription of the genes dependent on the particular factor involved will diminish. Unless single gene transcription factors can be identified, it is difficult to conceive how this approach, though potentially effective for controlling cell growth, can be made gene-specific. The polyamide approach may prove feasible since sequence-specific molecules can likely be designed and it appears that molecules of this type can easily access DNA within the chromosomes [23-25].

ANTI-MRNA STRATEGIES

A gene may be effectively "silenced" by destabilizing its mRNA, thereby preventing synthesis of the protein it encodes. Targeting mRNA, while less favorable than antigene strategies from a stoichiometric point of view, is nonetheless attractive because mRNA is in theory more accessible. Three basic strategies have emerged for this purpose. One employs an oligonucleotide that acts as an alternate binding site, or "decoy," for protein-stabilizing elements that normally interact with a given mRNA [26, 27]. By attracting away mRNA-stabilizing protein, the decoy induces instability, and ultimately destruction, of the mRNA. A newly developing approach is to affect RNA interference (RNAi) or post-transcriptional gene silencing [28, 29]. RNAi employs a gene-specific double-stranded RNA which, when introduced into a cell, leads to diminution of the targeted mRNA. The actual mechanism whereby this is accomplished is presently unknown but is under intense investigation with several clues being deciphered already [30, 31] including size and necessity for processing of the targeting dsRNA. In *C. elegans* and Drosophila this is a highly reproducible method for disrupting gene expression. Some reports suggest that this technique can be adapted for use in mammalian cells [32], but this remains uncertain at the moment. Finally, there is the more familiar, and more widely applied "antisense" strategy. We will focus on the latter.

Antisense (reverse complementary) nucleic acids are introduced into a cell in hopes that they will form Watson-Crick base pairs with the targeted gene's mRNA. As stated above, duplexed mRNA cannot be translated, and almost certainly initiates processes which lead to its destruction. The antisense nucleic acid can take the form of RNA expressed from a vector which has been transfected into the cell [33], or take the form of a DNA or RNA oligonucleotide which can be introduced into cells through a variety of means. DNA and RNA oligonucleotides can be modified for stability as well as engineered to contain inherent cleaving activity [34, 35]. A number of these issues will be discussed in more detail in the sections below.

Antisense Oligonucleotides (AS-ONs)

AS-ONs are short stretches of nucleotides that are complementary to a region of targeted mRNA and can specifically suppress expression of that particular transcript. The following discussion will focus on the fundamental concepts concerning AS-ONs and their mechanisms of action. Examples of AS-ON use in experimental and clinical settings have been recently reviewed [36-38].

The exact mechanism of AS-ON action remains unclear, but it is known to be different for various types of AS-ONs. Generally, these molecules block gene expression by hybridizing to the target mRNA, resulting in subsequent double-helix formation. This process can occur at any point between the conclusion of transcription and initiation of translation, or even possibly during translation. Disruption of splicing, transport, or translation of the transcripts are all possible mechanisms, as is stability of transcript. Therefore, a major question is whether AS-ONs are most effective in the cytoplasm or nucleus. In the case of antisense oligodeoxyribonucleotides (AS-ODNs), cellular RNase H is able to bind to the DNA-RNA duplex and hydrolyze the RNA, resulting in increased transcript turnover. Any modification to the deoxy moiety at the 2′-sugar position prohibits RNase H action.

Modified AS-ONs or AS-ON analogs are often employed for in vivo antisense applications due to their increased stability and nuclease resistance. A longer serum half-life ensures that the AS-ON has ample time to reach and interact with its target mRNA. Phosphorothioate AS-ODNs are most widely used due to their long serum half-life and the fact that they are a suitable RNase H substrate. However, phosphorothioates display high affinity for various cellular proteins, which can result in sequence-nonspecific effects [39, 40]. Furthermore, high concentrations of phosphorothioates inhibit DNA polymerases and RNase H, which may render them ineffective as antisense agents [41]. Interestingly, many AS-ONs with 2′-modifications with groups such as O-methyl, fluoro, O-propyl, O-allyl, or many others exhibit greater duplex stability with their target mRNA along with antisense effects independent of RNase H (Fig. 1). These modifications create bulk at the 2′ position, causing steric hindrance to play a significant role in increasing nuclease resistance. Nucleotide analogs

generally are also nuclease-resistant and often demonstrate superior hybridization properties due to modified backbone charge, although they usually are not acceptable substrates for RNase H. One example is peptide nucleic acid (PNA) where the sugar-phosphate moiety has been replaced by 2-aminoethyl glycine carbonyl units [42]. To these units are attached nucleotide bases spaced equally apart to DNA nucleotide bases. Instead of phosphodiester linkages between nucleotides, peptide bonds join the monomers to create a backbone neutral in charge. Not only do PNA oligomers hybridize to complementary DNA and RNA by Watson-Crick base pairing, they do so more quickly [43] and with greater affinity [42-44] because of the neutral backbone. In addition, PNAs are better at discriminating between base pair mismatches [44] and form less nonsequence-specific associations with proteins than phosphorothioate oligonucleotides [45]. Positive charges can also be introduced to backbone structure as in the case of (2-aminoethyl)phosphonates. Increased stability of duplex formation with both RNA and DNA has been reported with hybrid stability being more pH-dependent and less salt-dependent than natural RNA or DNA duplexes [46].

Some insight into the mechanism of AS-ON action has emerged recently through the work of *Baker* and colleagues (unpublished). Differences in ability to inhibit gene expression occur when either 2′-modified AS-ONs or 2′-unmodified AS-ONs are targeted to the exon 9 region of interleukin 5 (IL-5). Two forms of IL-5 exist: a soluble IL-5 lacking the exon 9 region, and a membrane-bound form, which contains exon 9. When unmodified AS-ONs are targeted to exon 9 of the IL-5 transcript, the expression of both membrane-bound and soluble IL-5 is inhibited. However, 2′-modified AS-ONs only suppress membrane-bound IL-5 expression. These observations seem to suggest that RNase H-dependent antisense effects are a nuclear event prior to splicing, whereas RNase H-independent oligonucleotides may affect splicing in transcript processing or may suppress gene expression after splicing has taken place. Additional evidence demonstrates that in the absence of RNase H activity, antisense effects may be a result of interference with translational initiation complex formation for certain types of 2′-modified AS-ON such as 2′-O-(2-methoxy) ethyl AS-ONs [47].

Ribozymes

Naturally occurring ribozymes are catalytic RNA molecules that have the ability to cleave phosphodiester linkages without the aid of protein-based enzymes. This property has been exploited to specifically inhibit gene expression by targeting mRNA for catalytic cleavage especially in viral, cancer, and genetic disease therapeutics [48]. Similar to AS-ONs, ribozymes bind to substrate RNA through Watson-Crick base pairing, which offers sequence-specific cleavage of transcripts. Ideally, these agents should trigger enhanced transcript turnover as compared to RNase H-mediated AS-ON degradation of transcripts, considering ribozymes act through bimolecular kinetics (association of ribozyme and target transcript) whereas RNase H-dependent AS-ONs rely on trimolecular kinetics (association of AS-ON, target transcript, and RNase H). Since ribozymes are RNase H-independent, 2′-modifications to increase stability do not diminish antisense effects and experiments have shown some modifications do not attenuate catalytic ability [49]. Unlike AS-ONs, ribozymes can be expressed from a vector, which offers the advantage of continued production of these molecules intracellularly [50, 51]. However, stable transformation of cells in vivo has its own complications and will not be discussed in this review.

If ribozymes are to perform effectively as "enzymes," they must not only bind substrate RNA but also dissociate from the cleavage product in order to act on additional substrates. Studies suggest that in some cases, dissociation of cleavage product may be the rate-limiting step [52, 53]. Furthermore, some ribozymes require high divalent metal ion concentrations for efficient substrate cleavage, which may limit their use in intracellular environments [54]. All of these concerns need to be addressed and overcome in order for ribozymes to have a future in medical therapy. Two ribozymes, the hammerhead ribozyme and the hairpin ribozyme, have been extensively studied due to their small size and rapid kinetics. Their application has been recently reviewed in several publications [55-59].

Hammerhead Ribozymes

The hammerhead ribozyme consists of a highly conserved catalytic core, which will cleave substrate RNA at NUH triplets 3′ to the H, where N is any nucleotide, U is uracile, and H is any nucleotide but guanidine (Fig. 2) [34]. In fact RNA cleavage may be less restricted since recent studies demonstrate exceptions to the "NUH" rule. Investigators have established that cleavage can actually occur 3′ to any NHH triplet [59]. Furthermore, in vitro selection protocols have made it possible to screen for ribozymes with various cleavage specificities including one that cleaves at AUG sites [60]. Thus, the limitations for sequence specificity of triplet-cleavage sites on the target RNA are less than previously thought. In addition to the catalytic core, a particular cleavage site in a target RNA can be specifically recognized by the hammerhead ribozyme arms. By creating complementary sequences in the arms to sequences flanking the cleavage site, the ribozyme will hybridize specifically to the RNA of interest.

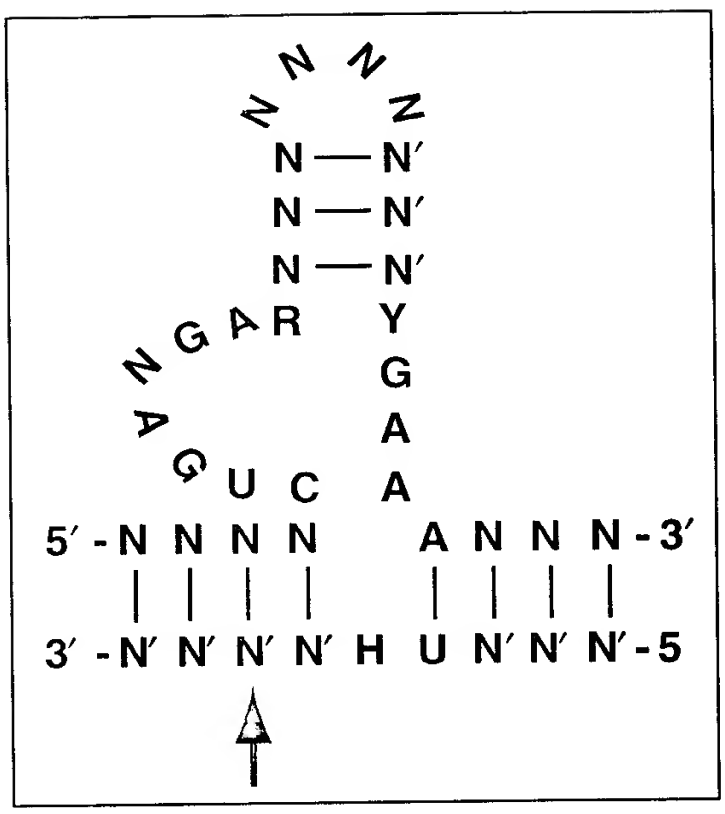

*Figure 2. **Hammerhead ribozyme (top strand) hybridized to target RNA.** Arrow indicates postition of cleavage. N = A, G, T, or C; N′ = nucleotide complementary to N; H = any nucleotide but G; Y = pyrimidine nucleotide; R = purine nucleotide complementary to Y. Adapted from [56].*

Subsequent cleavage will then be directed towards that particular position.

The catalytic ability of hammerhead ribozymes is dependent on the presence of divalent metal ions, of which magnesium is most often used in vitro. It is postulated that the ions not only participate in RNA folding but also in the cleavage step itself [54]. As mentioned previously, studies indicate that catalytic activity requires relatively high Mg^{2+} concentrations compared to the intracellular environment. This characteristic could be problematic in applying the hammerhead ribozyme to an in vivo setting where intracellular Mg^{2+} concentrations are 5- to 10-fold lower than optimal in vitro conditions.

Much evidence supports diminished mRNA levels and gene products directly due to hammerhead ribozyme delivery. There is also indication from reverse transcriptase-polymerase chain reaction (RT-PCR) and reverse ligation (RL)-PCR protocols of messenger RNA cleavage at the targeted position in cellular RNA extracts [61, 62]. Recently, more potent ribozyme-mediated effect on viral and cancer cell growth compared to noncatalytic RNAs was reported [63, 64]. However, in some instances, hammerhead ribozymes have not proven to be more effective than AS-ON and instead give equal degrees of gene suppression. Likewise, inactive control ribozymes where antisense binding can occur, but catalytic ability has been abolished, give similar levels of gene inhibition when compared to fully catalytic hammerhead ribozymes, suggesting that the catalytic core, in some instances, plays little role in enhancing antisense effects [65]. Only further detailed studies will reveal the true utility of hammerhead ribozymes.

Hairpin Ribozymes

The natural hairpin ribozyme is derived from a negative strand of the tobacco ringspot virus satellite RNA. Work on engineered hairpin ribozymes has resulted in a broader range of cleavage-sequence specificity. In general, a phosphodiester cleavage takes place 5′ to the G in the sequence NGUC where N is any nucleotide [66], although recent studies have shown even less restriction on sequence requirements for cleavage [67].

The overall structure of the hairpin ribozyme consists of two domains connected by a hinge section (Fig. 3). One domain binds the substrate RNA to form two helical regions separated by a pair of single-stranded loops. Cleavage occurs within the single-stranded area of the substrate RNA. The other domain is similar in structure except the helixes are formed from the ribozyme folding back onto itself. The most important sequences for cleavage activity are those within the single-stranded regions where almost every nucleotide is conserved, while the helical portions can be almost any sequence as long as there is double-helix formation [58]. The hinge allows the two domains to be flexible relative to one another in space so that the two can dock together in an antiparallel orientation required for cleavage catalysis [68, 69].

Both the hairpin and hammerhead ribozymes require metal ions for cleavage catalysis. In the hammerhead ribozyme, metal ions are believed to be involved directly in the cleavage step [54, 70], whereas metal ions have not been implicated to be directly involved with cleavage for the hairpin ribozyme [71]. The metal ions in hairpin ribozymes may instead play an important role in ribozyme structure [72]. Fluorescence resonance energy transfer (FRET) studies on docking of the two domains show that docking is metal-dependent, but almost any metal will suffice even though they may not support cleavage [73]. In addition, docking is not the rate-limiting step, and since metal ions are not thought to be involved in the chemical cleavage step, it can only be assumed that there is a slower step in between docking and chemical cleavage.

One of the advantages offered by hairpin ribozymes is their unique ion-dependence for catalytic action. One group has shown that aminoglycoside antibiotics with at least four amino groups are able to both support and to

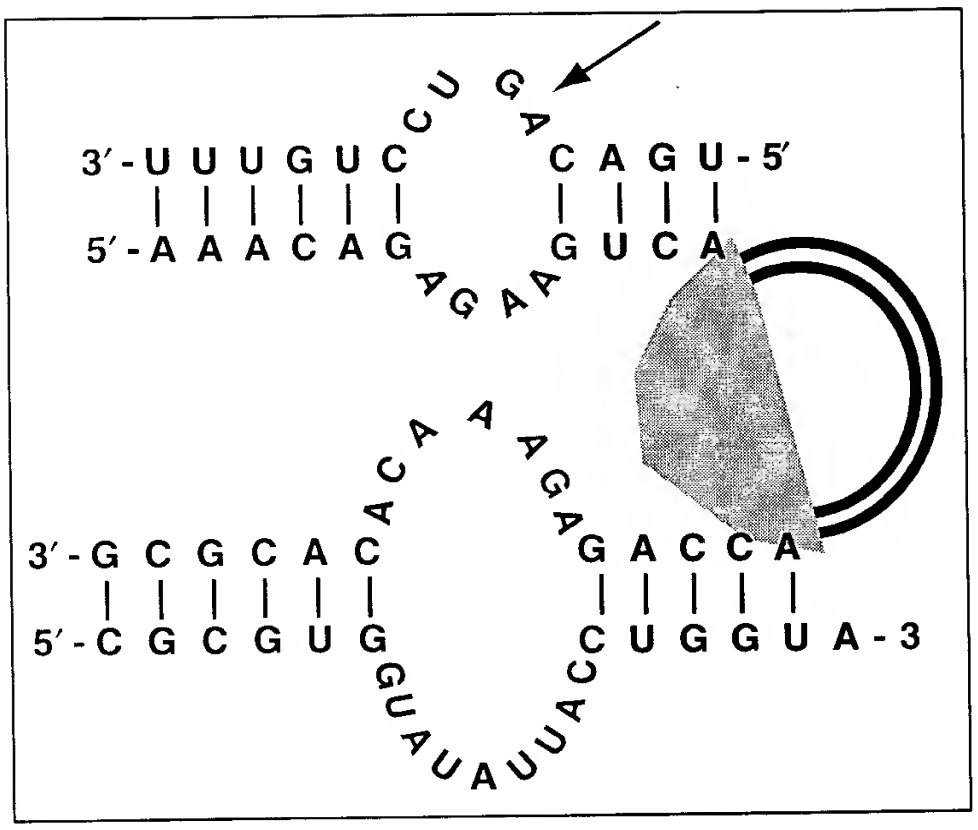

Figure 3. Hairpin ribozyme in the docked position. The two loop regions associate with each other in order to cleave the substrate RNA. Arrow indicates position of cleavage. Adapted from [58].

inhibit hairpin ribozyme cleavage depending on metal ion conditions [74]. In the presence of magnesium, aminoglycoside antibiotics inhibit ribozyme cleavage with the degree of inhibition depending on the binding affinity of the antibiotic to the ribozyme. However, in the absence of metal ions, aminoglycoside antibiotics prove to assist cleavage with an optimum reaction condition at pH 5.5 and poorer kinetics as the pH is increased, exactly opposite to trends observed for magnesium. In this case, the metal ions are most likely being replaced by the amino groups of these antibiotics.

Polyamines such as spermidine and spermine have also been reported to support hairpin ribozyme cleavage ability. In the absence of magnesium, spermidine allows the cleavage reaction to persist at very slow kinetics compared to magnesium alone [72]. However, spermine alone gives very efficient cleavage of RNA comparable to that of magnesium, and when in the presence of low magnesium concentrations similar to intracellular conditions, spermine displays considerable increase in cleavage rates [74]. The fact that spermine is the major polyamine in eukaryotic cells may explain why the hairpin ribozyme has shown remarkable intracellular cleavage activity in mammalian cells and may make future therapeutic endeavors with the hairpin ribozyme much easier [75].

DNAzymes

While investigating ways to improve the function of ribozymes, *Breaker* and *Joyce* made the assumption that because RNA and DNA are very similar chemical compounds, DNA molecules with enzymatic activity could also be developed [76]. This assumption proved correct and led to the development of a "general-purpose" RNA-cleaving DNA enzyme [77]. The molecule was identified from a library of >1,000 different DNA molecules by successive rounds of in vitro selective amplification based on the ability of individual molecules to promote Mg^{2+}-dependent, multiturnover, cleavage of an RNA target.

The selected molecule was named the "10-23 DNA enzyme," because it was derived from the 23rd clone obtained after the 10th round of selective amplification [77]. The "10-23 DNA enzyme" is composed of a catalytic domain of 15 deoxynucleotides, flanked by 2 substrate-recognition domains of ~8 nucleotides each (Fig. 4). The recognition domains provide the sequence information required for specific binding to an RNA substrate. They also supply the binding energy required to hold the RNA substrate within the active site of the enzyme. It is straightforward that by appropriately designing the flanking sequences, the DNAzyme can be made to cleave virtually any RNA that contains a purine-pyrimidine junction.

The attraction of DNAzymes over ribozymes is that they are very inexpensive to make and that because they are composed of DNA and not RNA, they are inherently more stable

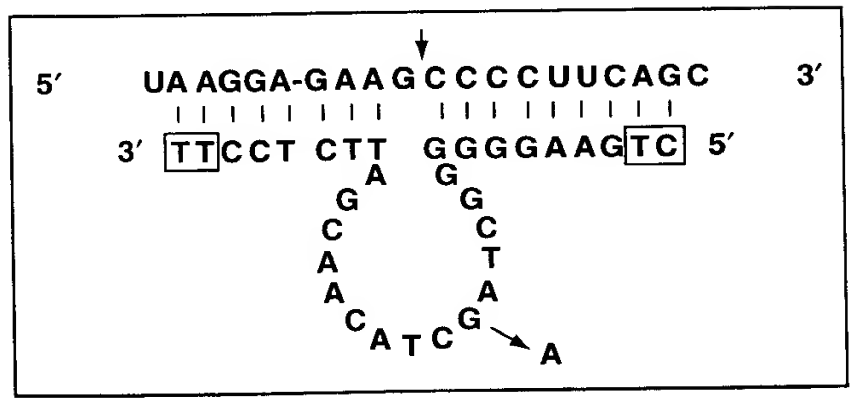

Figure 4. Complex formed by an mRNA (top strand) and a "10-23" DNAzyme (bottom strand). Vertical arrow indicates the mRNA cleavage site. Replacement of G by A within the catalytic core of the DNAzyme (diagonal arrow) will eliminate its catalytic activity. Adapted from [77].

than ribozymes. Nevertheless, DNAzymes must ultimately overcome the same problems faced by ribozymes and oligonucleotides if they are to be effective in cellular systems (see below). These are stability, ability to be targeted to the cell of interest, ability to achieve sufficient intracellular concentration to cleave to the targeted mRNA, ability to hybridize with their mRNA target, and lack of toxicity. In this regard, many of the chemical modifications employed to stabilize ODNs can be incorporated into the 10-23 DNA enzyme without loss of activity. There is a suggestion from recent reports that issues of intracellular concentration and target hybridization may also be solvable [78, 79].

Application of the "Antisense" Strategy

Although antisense interference methods appear impeccable in theory, many additional considerations must be taken into account in applying the strategy in living cells. Since both AS-ONs and ribozymes are considered oligonucleotides, quite often similar solutions can be offered to address the problems encountered. As mentioned earlier, increasing stability of antisense agents can be easily achieved through nucleotide modifications or analogs. However, additional considerations crucial to reliable experimental outcome include mRNA site selection, drug delivery, and intracellular localization of the antisense agent.

mRNA Site Selection

Within living cells, transcripts exist in low energy conformations in which secondary structures dominate in folding the linear polymer. In addition, interactions with cytoplasmic proteins produce further structural properties. The end result is that much of the mRNA sequence is hidden and only partial sequences within the total mRNA length are accessible for hybridization. RNA folding programs that generate three-dimentional folding patterns based on free energy calculations often give an unreliable depiction for in vivo relevance. Therefore, a good empirical method to probe for suitable sites is necessary.

A system to probe for suitable sites in mRNA for AS-ON or ribozyme-targeting has recently been established using RNase H cleavage as an indicator for accessibility of sequences within transcripts [80]. A mixture of ODNs that are complementary to certain regions of a transcript is added to cell extracts and exposed to RNase H. RT-PCR of the transcript can then be used to show which ODNs actually had access to the transcript and hybridized in order to create an RNase H-vulnerable site. Combining this methodology with computer-assisted sequence selection may enhance this approach as well [81].

Another technique currently being tested is the use of molecular beacons for site selection (*Gewirtz et al.*, unpublished). These molecules are ODNs with the ability to form stem-loops where the loops are targeted to regions of the transcript [82]. The stems have a fluorophore linked to either the 5′ or 3′ end and a quencher molecule is attached to the other so that in the stem-loop configuration, fluorescence is not observed due to the proximity of the quencher molecule to the fluorophore. However, when hybridization proceeds, the act of forming a double helix between the loop and the transcript causes unfolding of the stem-loop and brings the quencher and fluorophore apart in space. Thus, fluorescence should increase as a result of hybridization. Currently, these molecules are being applied to probe for accessible sites within mRNA with very encouraging results (*Jen* and *Gewirtz*, unpublished).

Delivery

One of the major limitations for the therapeutic use of AS-ONs and ribozymes is the problem of delivery. Import of these compounds into cells can be accomplished by exogenous delivery in which presynthesized oligonucleotides come in direct contact with the plasma membrane, resulting in subsequent cellular uptake [83]. Naked oligonucleotides are poorly incorporated into cells in this fashion and often require a vehicle for efficient delivery. In tissue culture, many classes of compounds have been used as delivery vehicles including cationic liposomes, cationic porphyrins, fusogenic peptides, and artificial virosomes. These compounds share the characteristic of forming complexes with oligonucleotides through electrostatic interactions between the negatively charged oligonucleotide phosphate groups and positive charges contained by the vehicles themselves. In addition, some degree of protection from nuclease degradation is conferred to the oligonucleotide when associated with such delivery vehicles. Other strategies including cell permeabilization with streptolysin-O and electroporation have been used [84] but are restricted in utility for clinical settings. Presently, some success has been achieved in tissue culture, but efficient delivery for in vivo animal studies remains questionable.

Cationic lipids form stable complexes with oligonucleotides, which exhibit improved cellular uptake [85-87]. The result is enhanced antisense activity. Further studies indicate that phosphorothioated ODNs dissociate from cationic lipids before entering the nucleus where it is free to hinder target transcript function [88]. These compounds have proven to be quite effective in cell culture and have been commercialized, but their relatively high cytotoxic properties may restrict their use.

Alternatives to cationic lipids are being explored. Recently, cationic porphyrins have proven to be effective vehicles for AS-ONs in tissue culture [89, 90]. Two cationic porphyrins used by *Benimetskaya* and colleagues, tetra(4-methylpyridyl) porphyrin (TMP) and tetraanilinium porphyrin (TAP), demonstrate properties important for AS-ON delivery. 5′-fluorescein-labeled phosphorothioates show that both TMP and TAP more efficiently deliver AS-ONs into cells than naked AS-ONs. Nuclear fluorescence is observed after porphyrin/AS-ON complex exposure to cells while fluorescein labeled AS-ONs alone are taken up into vesicular structures. Thus, cationic porphyrins not only help AS-ON delivery into the cell, but they are also able to localize the AS-ON in the nucleus where mRNA and RNase H are present. FRET studies on the ability of cationic porphyrins to quench 5′-fluorescein-labeled phosphorothioates suggest intracellular dissociation of the oligonucleotide from the porphyrin.

Fusogenic peptides form peptide cages around oligonucleotides in order to boost oligonucleotide uptake. Many of these peptides contain polylysine residues, which cause membrane destabilization [91]. Others are derived from viral proteins such as the fusion sequence of HIV gp41 [92] and hemagglutinin envelop protein [93, 94]. Generally, these agents are less cytotoxic than lipids but are still able to achieve similar delivery efficacy. Artificial virosomes are another class of delivery vehicles which take advantage of the natural ability of a virus to gain entry into cells. Reconstituted influenza virus envelopes known as virosomes can fuse with endosomal membranes after internalization through receptor-mediated endocytosis [95]. Recently, cationic lipids have been incorporated into virosome membranes to further aid delivery [96, 97].

Finally, *Dheur* and colleagues have noted that while oligonucleotides delivered with lipofectins usually do not elicit antisense activity (likely because cationic lipid formulations do not protect unmodified oligonucleotides from nuclease degradation), a cationic polymer, polyethylenimine (PEI) [98], improves the uptake and antisense activity of antisense phosphodiester oligodeoxynucleotides (PO-ODN) [99]. Interestingly, PEI-phosphorothioate (PS) ODN particles were efficiently taken up by cells but PS-ODN did not dissociate from the carrier. These investigators suggested that the low cost of PEI compared with cytofectins, the increased affinity for target mRNA and decreased affinity for proteins of PO-ODN compared with PS-ODN might make the use of PEI-PO-ODN very attractive.

Localization

In order for AS-ONs or ribozymes to suppress gene expression, they must be colocalized to the same intracellular compartment as their target mRNA. Intracellular trafficking seems to play an important role in the fate of these molecules since their spatial distribution does not correspond to simple diffusion. Many factors determine localization patterns of AS-ON and ribozymes including the antisense agent itself, delivery vehicle, and targeted cell type. In addition, evidence for cell cycle-dependent localization patterns has been reported with nuclear localization predominantly in the G_2/M phase [100].

mRNAs can exist in several cellular compartments including the cytoplasm, nucleus, and nucleolus. It remains unclear as to where oligonucleotides should be directed for most efficient antisense activity to occur, although endosomal localization usually predicts ineffective antisense response. The optimal site for mRNA degradation may be dependent on the type of antisense agent used [47]. Recently, ribozymes attached to small nucleolar RNAs (snoRNAs) called snoribozymes exhibited nearly 100% efficiency in cleaving a target RNA also localized to the nucleolus by snoRNA attachment [101]. Even though this particular experiment is based on cleavage of an artificial substrate, the expanding roles associated with the nucleolus may prove the nucleolus to be an important site to target mRNA degradation [102]. In another study, antisense RNA inserted within a variable region of ribosomal RNA (rRNA) proved to heighten ribozyme efficiency and may be due to colocalization of rRNA with mRNA [103].

Antisense Drug Design

Certain issues to be aware of concerning antisense experimental design are quite important to the consistent and efficacious outcome of inhibiting gene expression. Even when the above considerations regarding the potential problems of antisense experiments are addressed, other factors may come into play especially involving antisense drug design. Only two will be mentioned here: formation of G quartets and chirality of modified oligonucleotides. Purine-rich oligonucleotides, especially ones containing four consecutive guinine residues, have a tendency to form stable tetrameric structures under physiologic conditions [104]. The guinosines of single-stranded oligonucleotides are not restrained in space by rigid double-helix structure and can therefore form various hydrogen bonds not observed in Watson-Crick base pairing. Tetraplexes known as G quartets arise as a result. Dissociation rates of these structures may be quite slow and may prevent hybridization of AS-ONs or ribozymes to their target transcript, rendering them ineffective as antisense agents. However, the absence of G quartet structures at 37°C under cellular salt conditions could mean that G quartet formation is irrelevant at physiologic temperatures [105].

Interestingly, nonsequence-specific gene inhibition by phosphorothioate oligonucleotides containing tetraguinosine tracts prove aptameric properties can play an important role in gene inhibition for some sequences of ONs [106].

Another important aspect to consider is the issue of chirality for certain oligonucleotides. Unmodified phosphodiester oligonucleotides do not have a chiral center at the phosphorous position. However, when a terminal oxygen of the phosphate is replaced by a sulfur, as in PS-ONs, the phosphorous gains chirality. The digestion kinetics of PS-ONs by 3′-exonucleases display bi-exponential decay with a fast and slow phase of digestion. These phases are due to stereoselectivity of the 3′-exonucleases on the chiral phosphorothioate center [107]. A 25-mer containing a 3′-terminal internucleotide linkage in the S-configuration degrades 300-fold slower than the same 25-mer with an R-configuration phosphorothioate linkage.

Conclusions

The ongoing revolution in cell and molecular biology, combined with advances in the emerging disciplines of genomics and informatics, has made the concept of non-toxic, cancer-specific therapies more viable then ever. The recent development of a relatively specific biochemical inhibitor of the bcr/abl protein tyrosine kinase in patients with chronic myelogenous leukemia is a stunning example of this principle [108]. For therapies focused on direct replacement, repair, or disabling of disease-causing genes, progress has been much slower and a successful equivalent to the biochemical bcr/abl inhibitor has yet to be achieved. In the case of anti-mRNA strategies, it is hoped that the above discussion will have made the reasons for this clearer. Given the state of the art, it is perhaps not surprising that effective and efficient clinical translation of the antisense strategy has proven elusive. While a number of phase I/II trials employing ONs have been reported [109-116], virtually all have been characterized by a lack of toxicity but only modest clinical effects. A recent paper by *Waters et al.* describing the use of a bcl-2-targeted ON in patients with non-Hodgkin's lymphoma is typical in this regard [117, 118].

The key challenges to this field have been outlined above. It is clear that they will have to be solved if this approach to specific antitumor therapy is to become a useful treatment approach. A large number of diverse and talented groups are working on this problem, and we can all hope that their efforts will help lead to establishment of this promising form of therapy.

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ANNEX C

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HEPATOCELLULAR CARCINOMA

Effects of insulin-like growth factors-IR and -IIR antisense gene transfection on the biological behaviors of SMMC-7721 human hepatoma cells

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Abstract

Background and Aims: Insulin-like growth factors (IGFs) are closely related to hepatocellular carcinoma growth. The study aim was to investigate the effects of IGF-IR and IGF-IIR antisense gene transfection on the biological behaviors of SMMC-7721 human hepatoma cells.

Methods: 7721-IGF-IR-AS cells (human hepatoma SMMC-7721 cells transfected with IGF-IR antisense gene in our previous study) were transfected with a plasmid vector expressing IGF-IIR cDNA in the antisense orientation by DOTAP liposome. 7721-IGF-R-AS cells were obtained by selection with G418 and hygromycin. Morphological changes of the cells were observed with optic and electron microscopes. *In vitro* growth of the 7721-IGF-R-AS cells was observed with a soft agar test, MTT test and with naked mice inoculation test *in vivo*.

Results: The following changes were found in the SMMC-7721 cells after being transfected with the IGF-IR and IGF-IIR antisense genes: (i) the degree of malignancy of the tumor cells as revealed by cell morphology was ameliorated; (ii) the growth capability of the tumor cells in soft agar and their tumorigenicity in naked mice were significantly depressed. However, in the control groups, the SMMC-7721 cells transfected both with IGF-IR and IGF-IIR sense cDNA and SMMC-7721 cells transfected without any external genes, had no such changes. However, the cell growth curves had no significant differences among these three groups.

Conclusion: IGF-IR and IGF-IIR antisense genes could significantly restrain the malignant behavior of human hepatoma cells and might be useful in investigating a potential route for hepatocellular carcinoma gene therapy.

Key words: antisense gene; hepatocellular carcinoma, insulin-like growth factors-IR and -IIR.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in China. Clinical treatment for this cancer remains difficult because its pathogenesis is incompletely understood. With the rapid development of molecular biological theories and techniques, as well as advances in basic research related to HCC in recent years, new knowledge about molecular pathogenesis of HCC is continuously being revealed. In the recent years, more and more documents have shown that overexpression of insulin-like growth factors (IGFs) is one of the important reasons responsible for some tumor growths. Insulin-growth factor antisense gene transfection can inhibit growth of some tumors, such as prostate cancer, melanoma, neurolioma, colon cancer and breast cancer *in vitro*.[1–5]

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In our previous studies, single IGF-IR or IGF-IIR antisense genes were transfected into SMMC-7721 human hepatoma cells. The results showed that the proliferative ability of the transfected tumor cells was depressed and the transcription level of IGF-IR was significantly up-regulated after the IGF-IIR gene was obstructed, but there were no obvious differences in their cell growth curves.[6,7]

To our knowledge there have been no reports on what changes in biological behaviors will happen in HCC after both IGF-IR and IGF-IIR genes are simultaneously obstructed. Therefore, we performed such research with antisense techniques.

METHODS

Cell lines and culture

The human hepatoma cell line (SMMC-7721) was obtained from Shanghai Cell Research Institute of Chinese Scientific Academy, stored and transfer-cultured in our laboratory. SMMC-7721 cells transfected with IGF-IR sense or antisense genes in our previous experiment were named as 7721-IGF-IR-S and 7721-IGF-IR-AS cells, respectively. The transfection vector contained hygro-gene; 10% calf serum, 100 IU/mL penicillin and 100 IU/mL streptomycin in RPMI-1640 was used as conventional culture medium (400 μm/mL neomycin or 100 μm/mL hygromycin in the above conventional culture medium was used as selective culture medium). The culture procedures were taken under 37°, 5% CO_2 and saturation humidity.

Gene transfection

IGF-IIR sense and antisense expression vectors (constructed with pCMV plasmid-contained Neo gene) and IGF-IIR cDNA probes were obtained from Shanghai Tumor Research Institute, Shanghai Medical University. The experimental procedures of gene transfection were taken according to the directions of DOTAP™ liposome transfection kit. Forty-eight hours after the transfection, the cells were transfer-selection-cultured for 3 weeks under the pressure of 400 μm/mL G418 in the culture medium. Then the resistance cells expanded to be cultured under the selective pressure of 200 μm/mL G418 and 50 μm/mL hygromycin. The 7721-IGF-IR-S cells transferred with IGF-IIR sense eukaryon expression vector were renamed as 7721-IGF-R-S cells; whereas the 7721-IGF-IR-S cells transferred with IGF-IIR antisense eukaryon expression vector were renamed as 7721-IGF-R-AS cells.

Cell genome DNA extraction

Cell genome DNA extraction was performed according to the directions of Highpure™ plasmid extraction kit and Tripure™ nucleic acid extraction kit (Roche, Basel, Switzerland)

Polymerase chain reaction

A South2North™ HRP-random primer marker kit was purchased from Pierce Biotechnology, Inc. Rockford IL, USA. A PCR kit was purchased from Shanghai Promega Corproration, USA. A pair of IGF-IIR primers were designed by ourselves using commercial software (Primer Select 4.01) according to IGF-IIR cDNA sequence. The primers with upper stream in 3375 bp (P1-5 TGTTCCTTCTCCAGTGGACTG3), downstream in 4523 bp (P2-5 GACATAAGTCGGCA TCAACGT3), and its amplified product in 1148 bp length were synthesized by the Shanghai Bioengineering Company, Shanghai, China. The external gene can be specifically amplified because the sequence is long enough and spans at least one intron. 200 ng cell genome DNA extracted from 7721-IGF-R-S or 7721-IGF-R-AS cells was used as the template for polymerase chain reaction (PCR). The reaction system was as follows: template DNA 5 μL dNTP 2 μL, primer(P1+P2) 15 pmol 25 mM $MgCl_2$ 2.5 μL, 10× PCR buffer 2.5 μL Taq polymerase 1 U, sterile water to 50 μL in 0.2 mL reaction tube. After predegeneration at 95°C for 10 min in a PE cycler, 30 PCR cycles were performed at 94°C for 50 s, 60°C for 50 s and 72°C for 1 min, then extended to 10 min. The cell genome DNA extracted from 7721-IGF-IR-S or 7721-IGF-IR-AS cells was used as a negative control template.

Southern blot

The procedures of Southern blot for evaluation PCR amplified product were performed according to the methods in *Molecular Cloning*.[8]

Hematoxylin-eosin staining

Sterilized cover glasses were put into the holes of 6-hole plastic plates. After inoculation with the cells for 24 h, the glasses were then taken out of the holes, fixed in 10% formalin, stained with hematoxylin-eosin and observed under microscope.

Electron microscopy

After being bred into logarithm growth phase, cell suspension was obtained by digestion with a low concentration of pancreatin and repetitive blow-inhaling with a pipette. The cell suspension was transferred into a 5 mL glass centrifugation tube and centrifuged at 1200 g for 5 min. The upper liquid was removed. After being washed with saline once, the precipitation was fixed in 3% glutaric dialdehyde for electron microscopy.

MTT cell growth curve

This has been described elsewhere [6,7] The cells were cultivated for 8 days.

Double-deck soft agar colony forming efficiency

The cells were planted in 24-hole plastic plates spread with low melting-point agar (0.33% in upper layer and 0.5% in low layer). Each kind of cell was planted in five of the holes, with 2000 cells in each hole. The cells were cultivated under 37°C, 5% CO_2 and saturation humidity for 2-3 weeks. The cells with larger than 100μm in diameter or clones with more than 50 cells were counted under an invert microscope. Clone form-rate was calculated according to clone form number/inoculation cell number×100%.

Tumorigenicity in naked mouse

Six naked mice 4-5 weeks old (obtained from Animal Research Institute of The Third Military Medical University, Chongqing, China,) bred in specific pathogen free conditions were randomly divided into two groups. For each mouse, 4×10^5 SMMC-7721 cells were inoculated in the back of its left thigh as the control, while the same number of 7721-IGF-R-S or 7721-IGF-R-AS cells were inoculated in its opposite side. The tumor diameter was accurately measured every 7 days. The mice were killed after 6 weeks' observation. The tumor volume was calculated according to $L_x W^2/2$ (L, tumor length, W, tumor width).

Statistical analysis

Data were expressed as mean±standard deviation ($\bar{x}\pm SD$). Variance analysis and *t*-test for non-match data were performed by a professional SPSS statistical program.

RESULTS

Identification of transfection

As showed in Figure 1, the expect external DNA with 1148 bp in length could be specifically amplified using the template DNA from 7721-IGF-R-S or 7721-IGF-R-AS cells, but could not be amplified using the template DNA from control (7721-IGF-IR-S or 7721-IGF-IR-AS) cells. Expect bands could also be obviously observed in Southern blot film when an IGF-IIR DNA probe was used for hybridization. Therefore, IGF-IIR sense and antisense genes have been conducted into the 7721-IGF-R-S and 7721-IGF-R-AS cells, respectively.

Optic microscopy

Under an optic microscope, the cells with a larger body, larger nucleus and bigger ratio of nucleus/cytoplasm, were seen clearly in both 7721-IGF-R-S and SMMC-7721 cells. Multinuclei and karyokinesis were frequently seen in these two groups of cells, especially in the 7721-IGF-R-S cell group. Occasionally, the tumor giant-cells with multinuclei could be found in the 7721-IGF-R-S cell group. However, there were much smaller nuclei, a lower ratio of nucleus/cytoplasm and less frequent karyokinesis in the 7721-IGF-R-AS cell group (Fig. 2).

Figure 1 PCR amplified product in 1% gelose gel. Lane 1: DNA marker (1543, 994, 697, 515, 377, 237 bp). Lane 2: PCR product of 7721-IGF-R-S. Lane 3: PCR product of 7721-IGF-R-AS. Lane 4: PCR product of 7721-IGF-IR-S. Lane 5: PCR product of 7721-IGF-IR-AS.

Electron microscopy

The malignancy characteristics of 7721-IGF-R-S and SMMC-7721 cells were clearly demonstrated under an electron microscope with a higher nucleus/cytoplasm ratio, a larger nucleolus, multinucleolus, apparent microvilli and tumor giant-cells. However, the degree of malignancy of 7721-IGF-R-AS cells as revealed by morphology was significantly ameliorated. They exhibited a near normal ratio of nucleus/cytoplasm, a rounder nucleolus in shape, more regular nuclear membrane, fewer creases, but swelling and vacuole-formed mitochondria (Fig. 3).

Cell clonogenicity

7721-IGF-IR-S, 7721-IGF-IR-AS and SMMC-7721 cells were planted in 6-hole plastic plates, with 100 000 cells of one type only in each hole. The numbers of formed cell clones after transfection and selective culture with 400μm/mL G418 in its medium for 2-3 weeks, are listed in Table 1. The numbers of formed cell clones in the cells transfected both with IGF-IR and

Table 1 Number of G418 resistant cell clones formed after transfection

Cell type	Plasmid DNA (μg)	No. cells	No. transfection times 1	2	3	($\chi \pm SD$)
7721-IGF-R-AS	2.5	1×10^5	20	5	15	13.0 ± 7.5*
7721-IGF-R-S	2.5	1×10^5	36	40	28	34.7 ± 6.1
SMMC-7721	0	1×10^5	0	0	0	0 ± 0.0

*$P<0.05$ versus group 7721-IGF-R-S.

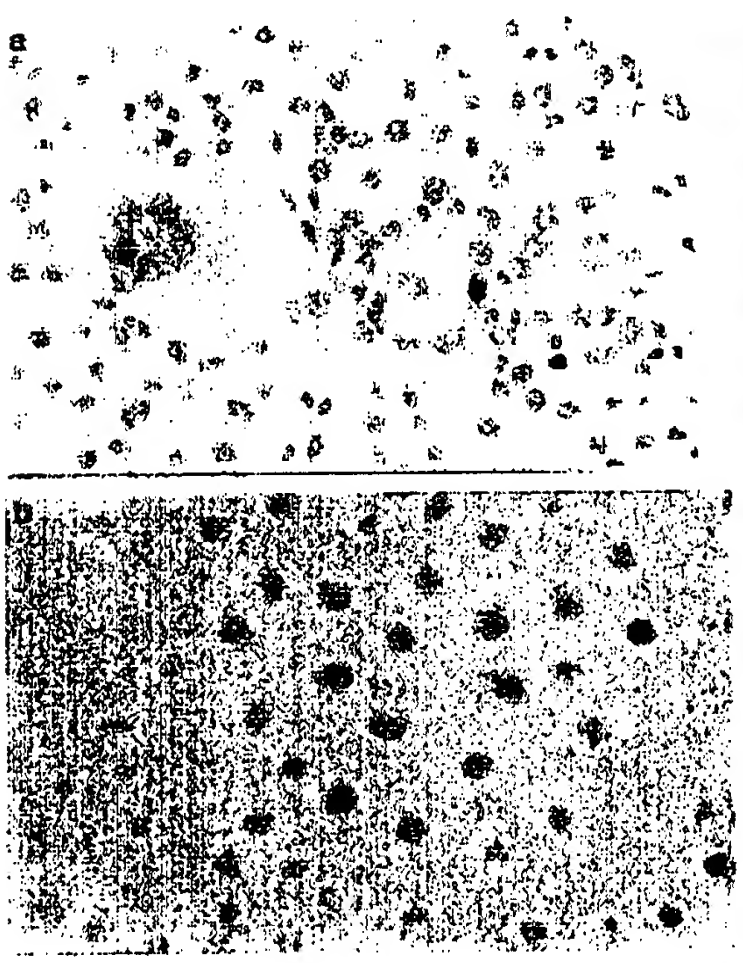

Figure 2 Cell morphology after transfecton (HE×200). (a) 7721-IGF-R-S; (b) 7721-IGF-R-AS.

IGF-II R antisense genes were significantly decreased as compared with the cells transfected both with IGF-IR and IGF-II R sense genes. This suggests that IGF-IR and IGF-II R antisense genes can inhibit cell clone formation of the tumor cells.

Cell growth curve

Figure 4 shows the cell growth curves of these three kinds of SMMC-7721 cells transfected both with IGF-IR and IGF-IIR sense genes, IGF-IR and IGF-IIR antisense genes, and without any external genes. No significant differences were noted among these three groups in 8 days' growth. This suggests there are no obvious effects of IGF-IR and IGF-IIR antisense genes in the 8-day growth curve of the tumor cells.

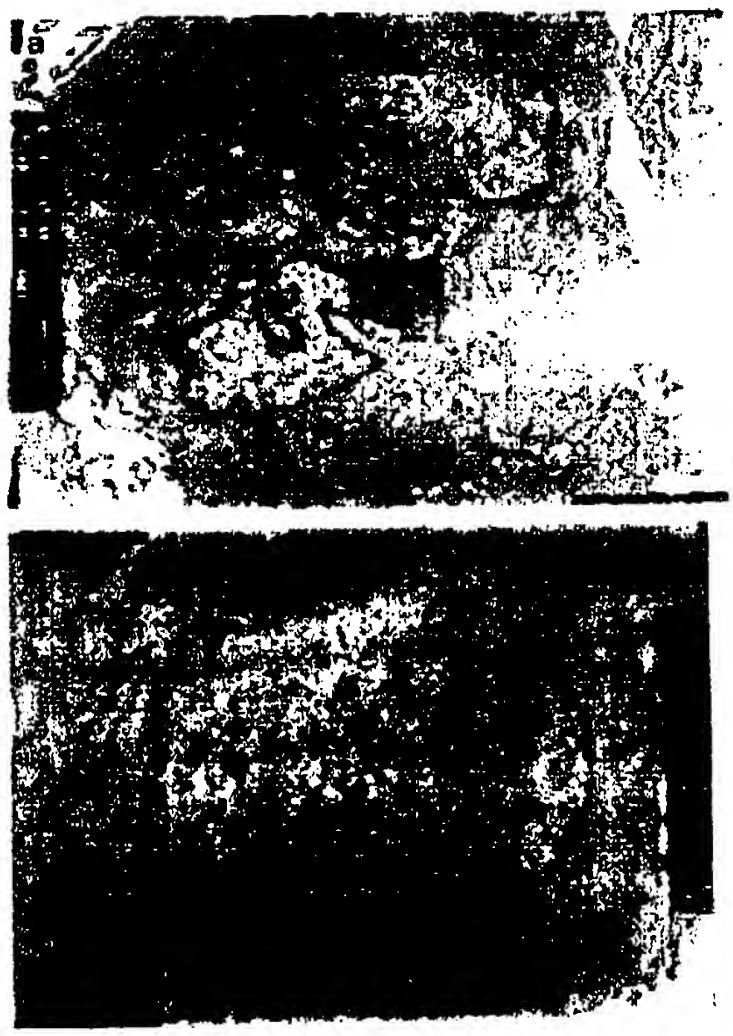

Figure 3 Cell morphology after transfecton (TEM×4000). (a) 7721-IGF-R-S. (b) 7721-IGF-R-AS.

Clonogenicity in soft agar

As show in Table 2 and Figure 5, in double-deck soft agar medium, SMMC-7721 cells multiplied more rapidly than the other two kinds of cells. Apparent cell clones usually began to form in days 5–7. Furthermore, the cell clones were larger in diameter and greater in number, with a clone formation rate of $10.29\pm0.85\%$. 7721-IGF-R-S cells increased more slowly than the SMMC-7721 cells. Cell clones were noted in days 7–10 and the clone formation rate was $8.25\pm1.75\%$. However, most of 7721-IGF-R-AS cells could not form cell clones in the soft agar medium and died after 2 weeks. Only a few cells formed smaller

Table 2 Effect of IGF-IR, -IIR antisense genes on SMMC-7721 clone formation in soft agar

Cell type	No. inoculation holes 1	2	3	4	5	$\bar{x} \pm SD$
SMMC-7721	230	190	212	208	189	205.8 ± 17.0
7721-IGF-R-S	218	17	159	140	130	165.0 ± 84.9
7721-IGF-R-AS	9	7	2	0	0	3.6 ± 4.2*

Cells were seeded at a concentration of 2000 cells per hole and the number of colonies were calculated after 3 weeks. *$P < 0.01$ versus group SMMC-7721 or group 7721-IGF-R-S.

Table 3 Effect of IGF-IR, -IIR sense and antisense gene on tumor inducation in naked mice

Cell type	No. cells (1×10^5)	Growth of tumor: Frequency	Days of appearance	Tumor volume (mm^3) ($\bar{x} \pm SD$)
Cell type 1				
7721-IGF-R-AS	4	2/3	25	35.2 ± 26.7*
SMMC-7721	4	3/3	17	183.5 ± 18.6
Cell type 2				
7721-IGF-R-S	4	3/3	16	227.7 ± 81.2**
SMMC-7721	4	3/3	17	220.7 ± 30.6

*$P < 0.05$; **$P > 0.05$ versus group SMMC-7721 (on the opposite side).

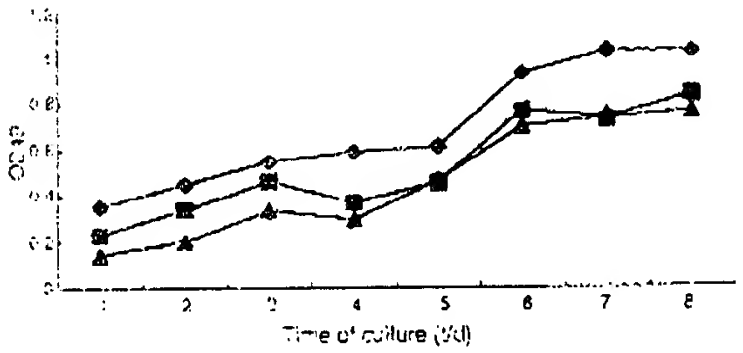

Figure 4 Effect of IGF-IR,IIR antisense gene on SMMC-7721 cell growth. (◆), 7721-IGF-R-S; (■) 7721-IGF-R-AS; (▲) SMMC-7721. t/d, time per day.

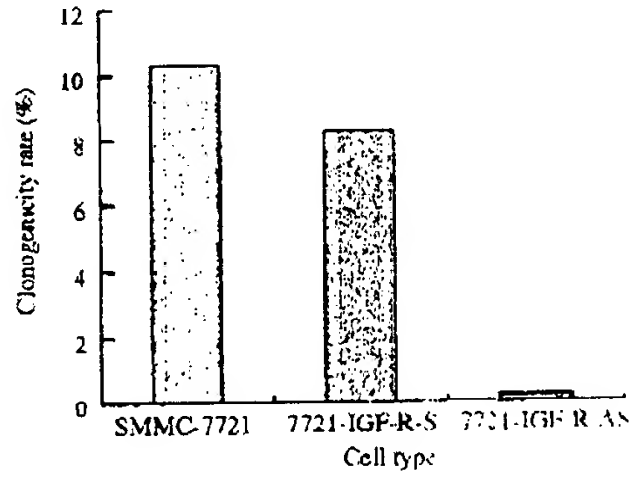

Figure 5 Clone formation rate of SMMC-7721,7721-IGF-R-S and 7721-IGF-R-AS growing in soft agar.

cluster-clones and the clone formation rate was only 0.18±0.21%.

Tumorigenicity in naked mice

As show in Table 3, the migrated tumors inoculated with 7721-IGF-R-S or SMMC-7721 cells could be found after inoculation for 16–17 days. But the migrated tumors inoculated with 7721-IGF-R-AS cells could not be found until day 25. Morcover, the mean migrated tumor volume inoculated with 7721-IGF-R-AS cells was much smaller than those inoculated with SMMC-7721 cells in the opposite side or those inoculated with 7721-IGF-R-S cells in the control group ($P < 0.05$). This indicates that the malignancy degree and tumorigenicity of the tumor in naked mice decreased significantly after IGF-I-R and IGF-II-R antisense gene transfection.

DISCUSSION

Some authors have reported that the IGF function in stimulating cell proliferation occurs mainly through the IGF-IR route. But others consider that IGF-IIR also plays a certain role in cell growth through influencing ion or matrix transport and increasing the synthesis of

RNA, protein and glycogen.[9] Other researchers have proposed that there may exist an autocrine and paracrine system in IGF genes to promote cell proliferation in the tumor itself, because simultaneous overexpression of the IGF-II gene and its receptor IGF-IR, IGF-IIR can be noted in human HCC and its surrounding tissues.[10,11]

In this study, we transfected both IGF-IR and IGF-IIR antisense genes into SMMC-7721 cells simultaneously, which inhibited the expression of both IGF-IR and IGF-IIR genes in the tumor cells. The results showed that the number of G418 resistant cell clones formed after transfection both with IGF-IR and IGF-IIR antisense genes was significantly less than those transfected both with IGF-IR and IGF-IIR sense genes. The malignancy degree of the tumor cells transfected both with IGF-IR and IGF-IIR antisense genes, revealed by optic and electron microscope, was also significantly ameliorated as compared with the original cells and the cells transfected both with IGF-IR and IGF-IIR sense genes. The growth capability of the tumor cells transfected both with IGF-IR and IGF-IIR antisense genes in soft agar and their tumorigenicity in naked mice were also significantly decreased. These data suggest that IGF-IR and IGF-IIR genes are among the important genes in maintaining the malignant phenotypes of the SMMC-7721 cell line. The phenotypes of the tumor will be restrained when these two genes are inhibited.

However, no significant difference in the 8-day cell growth curve was noted among these three groups in the present research. In our previous studies, we also found transfection with single IGF-IIR antisense gene did not significantly effect the 8-day growth curve of SMMC-7721 cells.[7] Although single IGF-IR antisense gene transfection had a small effect on the growth curve of SMMC-7721 cells, the inhibition could only be observed 8 days later.[6] This may be caused by: (i) the growth of cells being controlled and regulated by many cellular genes. Compensative activation or enhancement expression of other cellular genes, such as insulin, colony stimulating factor-IR(CSF-IR), transforming growth factor-ß1(TGFß1) and epidermal growth factor receptor (EGFR) may happen when the function of IGF genes is inhibited so as to antagonize the restraining effects of IGF-IR and IGF-IIR genes;[12] (ii) compensative enhancing expression of human insulin receptor (HIR) may result in reverse action. The biological function of IGF-I gene depends on combining with IGF-IR, IGF-IIR and HIR. IGF-I gene can unceasingly play its role in promoting cell growth by HIR when IGF-IR and IGF-IIR are inhibited; and (iii) different degrees of IGF-IR,IIR gene restraint may have different biological effects. Rubini *et al.* has found the number of IGF-I receptors is connected with the IGF-I-mediated mitogenesis and transformation of mouse embryo fibroblasts.[13]

In conclusion, we simultaneously transfected both IGF-IR and IGF-IIR antisense into SMMC-7721 cells and observed the morphological and biological changes of the tumor cells. The results reveal that IGF-IR and IGF-IIR antisense gene transfecton can partly reverse malignant behaviors of SMMC-7721 cells. This finding may be helpful in researching a potential route for hepatocellular carcinoma gene therapy in the future.

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Annex D

Stable expression of antisense urokinase mRNA inhibits the proliferation and invasion of human hepatocellular carcinoma cells

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Urokinase-type plasminogen activator (u-PA) plays a key role in malignant tumor behavior. We have previously shown that the expression of high levels of u-PA mRNA in human hepatocellular carcinoma (HCC) biopsies was inversely correlated with the survival of the patients. In order to evaluate the involvement of u-PA in the invasive and infiltrating properties of HCC cells, the SKHep1C3 cell line was stably transfected with an expression vector containing the 5' portion (257 bp) of u-PA cDNA in the antisense orientation. u-PA mRNA expression and its protein level and enzymatic activity were specifically inhibited in the antisense transfectants. A comparable inhibition of the u-PA receptor (u-PAR) mRNA and protein was also evidenced in the antisense transfected cells compared with the control ones. At the functional level, the SKHep1C3-AS cells showed a significant reduction in proliferation, Matrigel invasion, and motility assays compared to parental and vector-alone cells. These results indicate that u-PA is an essential factor in the growth and invasiveness of human hepatocarcinoma cells. Antisense u-PA strategy might be a potential approach to reduce tumor growth as well as the invasive capacity of the malignant cells in HCC. Cancer Gene Therapy (2003) 10, 112-120 doi:10.1038/sj.cgt.7700533

ANNEX E

Oncogene (2003) 22, 2405–2416

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Delivering antisense telomerase RNA by a hybrid adenovirus/adeno-associated virus significantly suppresses the malignant phenotype and enhances cell apoptosis of human breast cancer cells

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Activated telomerase is frequently detected in cancer cells and is able to maintain and stabilize the integrity of telomeres; it also contributes to unlimited divisions in cancer cells. Recently, a new generation of selective anticancer strategies is under development targeting the blockage of telomerase activity either at the protein level or telomerase RNA. Here, we report suppression of the malignant phenotype by the expression of the full-length antisense human telomerase RNA (hTR) delivered by a novel hybrid vector recombining adenovirus and adeno-associated virus (vAd–AAV). The hybrid vector vAd–AAV retained the unique traits from two parental viruses, such as high efficiency of gene transfer in mammalian cells and the ability to integrate into the genomic DNA of host cells. The stable expression of antisense hTR in MCF-7 cells significantly suppressed telomerase activity and progressively shortened telomere length for 30 population doublings (PD30). Expression of antisense hTR leads to a telomere-based growth arrest and the induction of spontaneous apoptosis. Antisense hTR decreased soft agar colony formation and reduced the cell proliferation, leading to exit from the cell cycle at G1 at PD15. The expression of antisense hTR also sensitized MCF-7 cells to apoptosis induced by sodium butyrate or serum starvation. Our study demonstrates that delivering antisense hTR by the hybrid Ad/AAV vector is an effective antineoplastic gene therapeutic strategy, which significantly suppresses the malignant phenotype and enhances apoptosis of human breast cancer cells.
Oncogene (2003) **22**, 2405–2416. doi:10.1038/sj.onc.1206317

Keywords: telomerase RNA; antisense; adenovirus; adeno-associated virus; gene therapy; breast cancer

Introduction

The malignant phenotype in cancer cells is characterized by unlimited cell proliferation and resistance to programmed cell death (apoptosis). One of the essential mechanisms governing these processes is mediated by the integrity of telomeres, the natural ends of linear chromosomes. Human telomeres are 10–15 kb chromosomal DNA composed of tandem hexameric repeats, 5′-TTAGGG-3′, which are maintained and stabilized by telomere-binding protein, and in stem cells and cancer cells by telomerase activity. In most normal cells, telomerase is not expressed and telomere DNA continues to shorten with each cell division (Kim *et al.*, 1994; Prowse and Greider, 1995; Holt *et al.*, 1997). Continued shortening of telomere DNA eventually triggers the cells to enter a growth arrest/senescence stage, and the proliferative lifespan of the cells will be terminated (reviewed by Chiu and Harley, 1997; Shay and Wright, 2001). Such a cellular mechanism ensures normal cells only to proliferate within a limited number of passages, and cell senescence/apoptosis will be enforced when the programmed cell lifespan ends. This may have evolved in large, long-lived species as a potent anticancer protection mechanism. However, in tumor cells, cell senescence/apoptosis may not be induced by the crisis triggered by shortened telomeres, because of aberrant status of other critical cellular regulatory mechanisms, such as p53, Rb, p16INK4a, or other pathways (Di Leonardo *et al.*, 1994; Robles and Adami, 1998; reviewed by Campisi *et al.*, 1996, 1999; Wright and Shay, 1996). A very rare cell undergoing crisis can reactivate telomerase and the cell is then able to restore the length of the telomeres or compensate for the progressive telomere shortening after each cell division. The stabilization of telomere length allows these cells to escape from the crisis stage of apoptosis and continues to proliferate. In addition, the crisis situation induces genome instability, which leads to gene mutations or deletions (Hahn *et al.*, 1999a, b). It has been hypothesized and demonstrated by *in vitro* experiments that malignant transformation can be established when normal cells acquire mutated oncogenes, defective tumor-suppressor genes, and activated

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telomerase (Hahn *et al*., 1999a, b; Gonzalez-Suarez *et al*., 2001).

Human telomerase is a multiple subunit-containing ribonucleoprotein enzyme. The core subunits are comprised of an RNA component (human telomerase RNA, hTR) and a protein reverse transcriptase (human telomerase reverse transcriptase, hTERT). The RNA component, hTR, serves as the template for synthesis of telomeric repeats (Feng *et al*., 1995). hTR is ubiquitously expressed, but levels of hTR in normal adult cells vary and correlate with cell activation status (Avilion *et al*., 1996; Bodnar *et al*., 1996). Immortalized cancer cells retain the highest level of hTR (Mitchell *et al*., 1999). The protein component, hTERT, uses hTR as the template to cap the termini of chromosomes, reverse transcribes RNA sequence to telomeric DNA, and adds these sequences onto the ends of chromosomes (reviewed by Collins and Mitchell, 2002; Ducrest *et al*., 2002). hTERT expression is more restrictive in its expression and closely correlates with telomerase activity (reviewed by Collins and Mitchell, 2002; Ducrest *et al*., 2002). Both components are necessary for effective telomerase activity *in vivo* (Counter *et al*., 1992; Wright *et al*., 1996; Niida *et al*., 1998; Mitchell *et al*., 1999; Mitchell and Collins, 2000). Targeting hTR and hTERT has been hypothesized as potential novel anticancer strategies, and *in vitro* proof of principle has been demonstrated in several cancer cell lines (Feng *et al*., 1995; Blasco *et al*., 1997; Kondo *et al*., 1998; Hahn *et al*., 1999b; Herbert *et al*., 1999). However, few effective delivery strategies have been reported to target hTR in cancer cells, and the potential use of hTR-targeted gene delivery systems to enhance effects of cell apoptosis-inducing agents need to be tested.

Different gene delivery systems have been developed in recent years to alter gene expression by specific targeting of DNA or RNA molecules. Our laboratory has developed an effective gene delivery hybrid vector combining adenovirus (Ad) and adeno-associated virus (AAV) to transfer and express antisense molecules in tumor cells (Wang *et al*., 1997a). The hybrid vector retains the advantages of both virus vectors by conferring high transfection efficiency and the capacity to integrate exogenous DNA into host genomic DNA (Wang *et al*., 1997a). Adenoviral vectors are frequently used for gene therapy because of their high transduction efficiency *in vitro* and *in vivo* (reviewed by Ali *et al*., 1994; Douglas, 1994). The development of a gutless-Ad vector through deletion of viral genes offers the advantages of decreased host immunogenicity and cellular toxicity induced by viral proteins, and increased capacity to accommodate large regulatory and exogenous DNA regions (reviewed by Kochanek, 1999). AAV is a member of the *Parvoviridae* family, which requires adenovirus coinfection for efficient gene expression, replication, and propagation (reviewed by Smith and Kotin, 2002). It has been found that AAV is able to stably integrate its DNA into the host genome with high efficiency, usually occurring at a specific site on chromosome 19 (Kotin *et al*., 1990, 1991, 1992). The high frequency of site-specific integration by AAV and the lack of associated disease have made AAV a promising vector for gene therapy. However, the unfavorable features of AAV in gene therapy are its low productivity and lack of ability to carry large segments of exogenous DNA. In recent years, considerable progress has been made to improve the vectors for more effective gene transfer, one being the development of hybrid Ad/AAV vector. The construction of hybrid Ad/AAV vector is achieved by the deletion of viral genes from both Ad and AAV to produce a DeltaAd/AAV vector that contains only the transgene flanked by AAV inverted terminal repeats (ITRs), Ad packaging signals, and Ad gene (Lieber *et al*., 1999; Recchia *et al*., 1999; Shayakhmetov *et al*., 2002). The DeltaAd/AAV hybrid vector has been shown to transduce cultured cells at the efficiency comparable to recombinant AAV vectors, and the hybrid vector DNA was integrated as head-to-tail tandem into the host cell genome (Wang *et al*., 1997a).

In this study, we utilized the novel hybrid recombinant vector from Ad and AAV to express antisense RNA to hTR in MCF-7 breast cancer cells. Using this vector, we successfully delivered antisense hTR into MCF-7 breast cancer cells and established MCF-7 cell lines stably expressing antisense hTR. Expression of antisense hTR in MCF-7 cells blocked telomerase activity and progressively shortened telomere DNA over 30 population doublings (PD30). The malignant phenotype of MCF-7 cells was significantly suppressed, with decreased colony-forming ability and cell proliferation rate, and blockage of the cell cycle in the G1 phase. The antisense hTR-expressing cells appeared more sensitive to apoptosis induced by chemical agents or serum starvation. Our study demonstrates that targeting hTR using this gene therapy strategy in cancer cells is efficient and powerful, and the new vector system may allow for the development of new generations of therapeutic agents.

Results

Hybrid vector vAd–AAV retained the capabilities of high infection efficiency, host genome integration, and stable expression of antisense hTR in MCF-7 cells

The newly developed hybrid vectors retained high infection efficiency similar to the parental E1/E3 deleted Ad (Wang *et al*., 1997a). The inclusion of AAV ITRs conferred upon the new vectors the ability to integrate into the host genomic DNA (Figure 1). Figure 2 shows a Southern blot analysis using genomic DNA extracted from parental MCF-7, and MCF-7 cells infected with either control vector or antisense hTR vector (MCF-7/control and MCF-7/anti-hTR), at PD5 after the stable cell lines were established. The integration of viral DNA was detected by analysis using Neo probe (Figure 2a) or hTR cDNA probe (Figure 2b and c). After *Xba*I digestion, Neo probe detected the predicted 3.16 kb band in the genomic DNA of MCF-7/control cells, and the 3.76 kb band in the genomic DNA of MCF-7/anti-hTR, but no band was detected in the DNA isolated

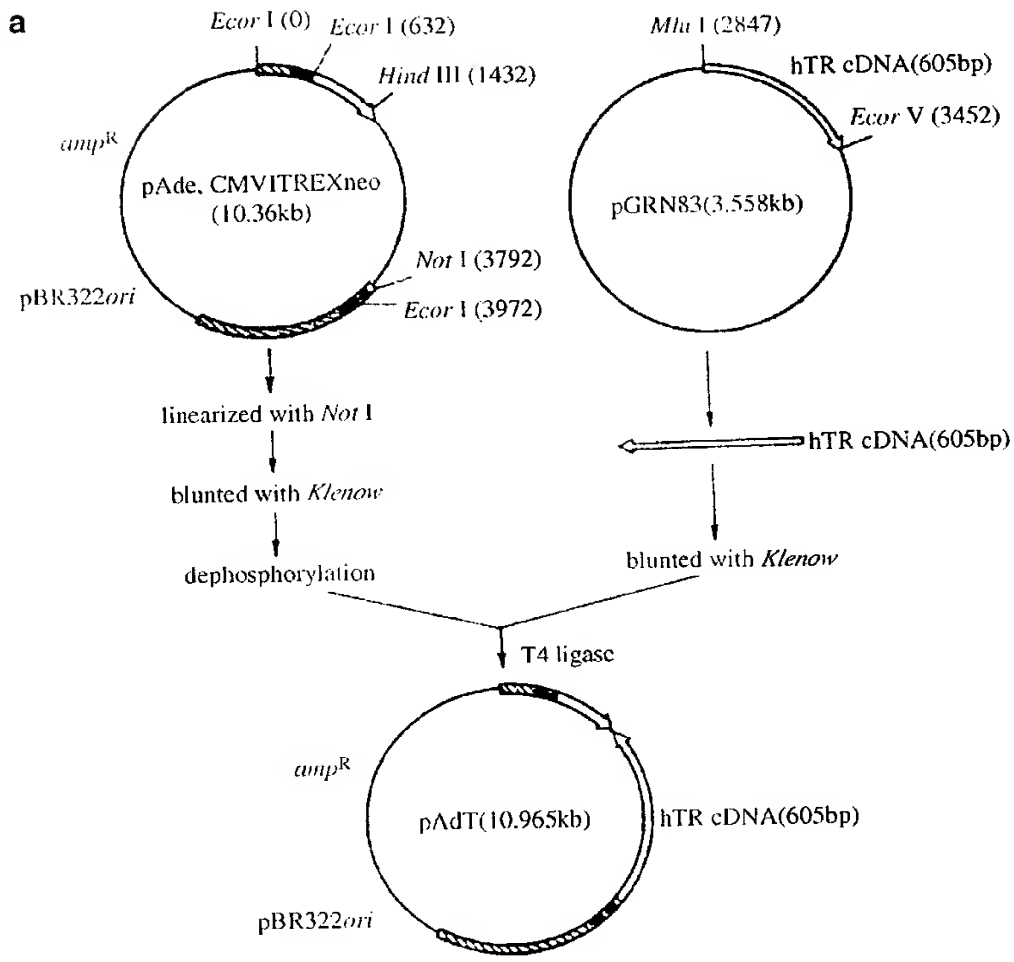


Figure 1 Plasmid construction of vAd–AAV hybrid vector encoding antisense hTR. (a) pAdE$_1$CMVITREXneo is a novel vAd–AAV hybrid vector with capacity to integrate foreign gene into genome of host cells. pAdT (vAd–AAV hybrid vector encoding antisense hTR) is engineered by inversely integrating hTR cDNA (605 bp, from pGRN83 plasmid) into the vector of pAdE$_1$CMVITREXneo. **(b)** elements of virus genome in the vAd–AAV hybrid vector encoding antisense hTR. The upper one is the map of empty control vAd–AAV hybrid vector. From the left: the dashed bar: the left boundary of adenovirus left arm, 0–1.26 mu, 452 bp; two black arrows pointing left: AAV ITR (inverted teminal repeat) 180 bp; white bar with arrow: CMV promoter; fine dotted bar: neo gene; bold dotted bar: SV40 promoter polyA tail; two black arrows pointing right: AAV ITR, 180 bp; and dashed bar: adenovirus right ITR, 2.5 kb, 9.2–16.0 mu. The lower one depicts the hybrid vector with antisense hTR, which has been inserted between SV40 polyA tail and the second AAV ITR (white open bar)

from parental MCF-7 cells. The hTR cDNA probe detected the correct 3.76 kb band in genomic DNA isolated from MCF-7/anti-hTR using the restriction enzymes *Xba*I (Figure 2b), and the 2.95 kb band digested by *Hind*III + *Pst*I (Figure 2c), respectively. No band was detected in the DNA from parental MCF-7 and MCF-7/control cells (Figure 2b and c), confirming the specificity of the detection. Our data demonstrated that the hybrid

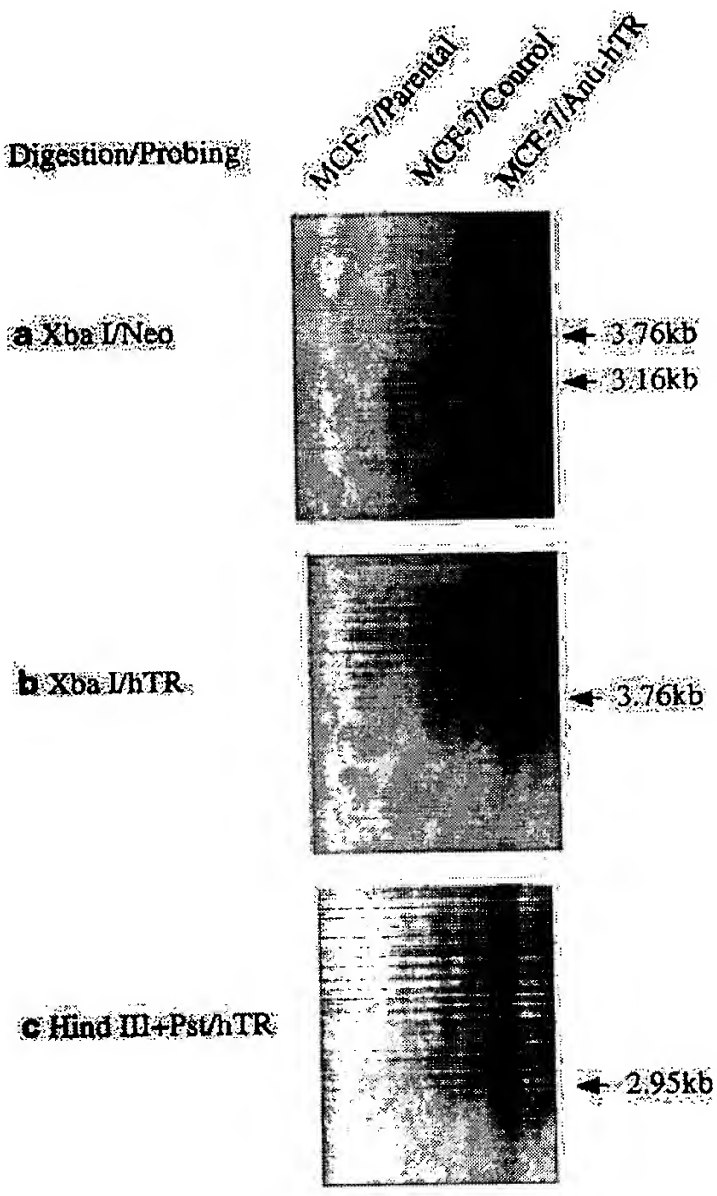


Figure 2 vAd–AAV hybrid vector is able to integrate antisense hTR into MCF-7 genomic DNA. The genomic DNAs were isolated from MCF-7 cells infected with control vAd–AAV or vAd–AAV encoded with antisense hTR and examined by Southern blots analysis. The DNA samples were loaded on the gel from left: MCF-7/parental, MCF-7/control, and MCF-7/antisense hTR. (**a**) The genomic DNAs were digested with *Xba*I and detected with neo gene probe. (**b**) The genomic DNAs were digested by *Xba*I and detected with hTR probe. (**c**) The genomic DNAs were digested by *Hind*III + *Pst*I and detected with hTR probe

vectors of vAd–AAV were able to integrate exogenous hTR antisense DNA into the host genomic DNA. Antisense hTR expression in MCF-7-transfected cells was monitored by Northern blot analysis at PD5 and PD20 (Figure 3). At both PDs, the comparable higher expression level of antisense hTR was observed in the MCF-7/anti-hTR cells.

Antisense hTR inhibited telomerase activity and shortened telemere length in MCF-7 cells

Telomerase activity was tested by PCR-based telomere repeat amplification protocol (TRAP assay) in the cells infected with the hybrid virus at PD10, 20, and 30 with the different titers of virus. As shown in Figure 4a, a complete loss of the laddering PCR bands in higher

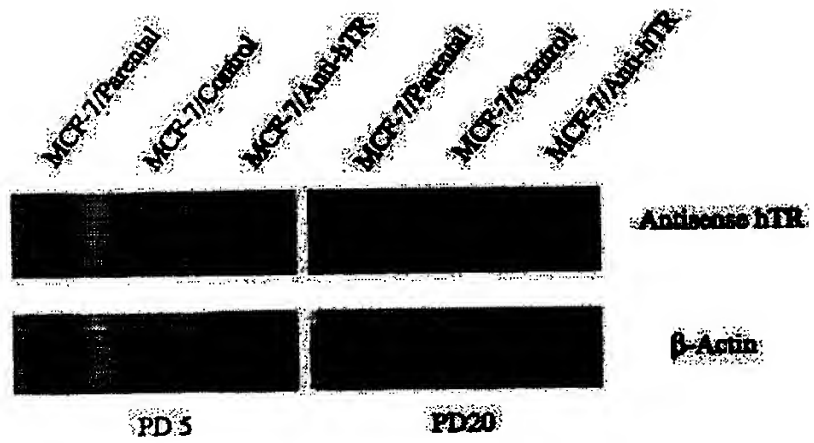


Figure 3 Antisense hTR expression in MCF-7 cells. hTR cDNA probe was used to detect antisense hTR expression was detected by Northern blot analysis of the total RNA isolated from MCF-7/parental, MCF-7/control, and MCF-7/anti-hTR cells at PD5 (left panel) and PD20 (right panel). As the internal control for the amount of total RNA loading, the same blots were stripped and probed with β-actin DNA probe

molecular weight range was observed in MCF-7/anti-hTR cells at PD30, as compared with MCF-7/control and MCF-7/parental cells. The telomerase activity in antisense hTR-expressing MCF-7 cells was also significantly suppressed when measured at PD10 and PD20, the suppression was at the similar level as in the TRAP assay of PD30 (data not shown). This indicates that the shortened telomeres in MCF-7/anti-hTR cells are likely because of the suppression of telomerase activity. The shortened telomeres in MCF-7/anti-hTR cells were further confirmed by testing the terminal restriction fragments (TRFs) in cells from PD15 and PD30. As shown in Figure 4b, TRFs appeared as a smear on the gel because of the broad distributions of both the sizes of the subtelomeric region and the number of TTAGGG repeat on different chromosomes. At PD15, no significant difference was observed in the size distribution and the DNA density of TRFs isolated from parental, control, and anti-hTR virus-infected MCF-7 cells (Figure 4b). However, at PD30, significant shortened telomeres (mean = 7.28) were observed in MCF7/anti-hTR cells when compared with the control and parental cells (means = 9.10 and 9.25). Furthermore, a decrease in band intensity (representing less amount of TRFs) was observed in MCF7/anti-hTR cells in PD30 (Figure 4b).

Antisense hTR significantly suppressed the transformation and malignant growth phenotypes of MCF-7 cells

Stable expression of antisense hTR in MCF-7 cells significantly suppressed their transformation and malignant growth phenotypes, including colony formation in soft agar, proliferation rate, and cell cycle distribution. Antisense hTR-suppressed cell transformation ability was measured by soft agar colony-forming assay (Table 1). At PD20, significantly less colony numbers were observed in MCF-7/anti-hTR cells when compared with controls, and the larger difference was observed at PD30 (Table 1). The significantly smaller colony sizes of

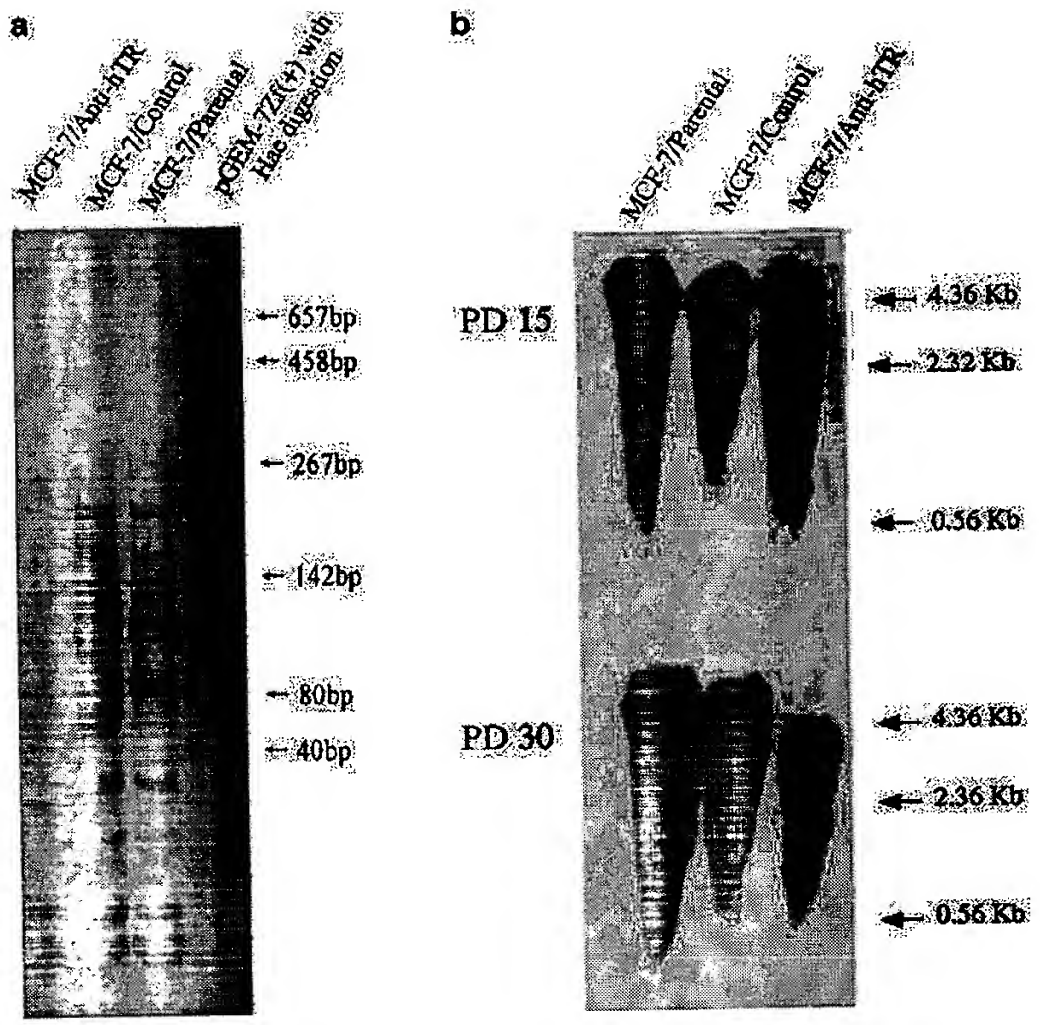


Figure 4 MCF-7 cells infected with antisense hTR virus vector showed decreased telomerase activity and shortened DNA telomeres. **(a)** The PCR-based TRAP assay was used to determine telomerase activity in cells after 30PD. The gels of electrophoresed PCR products were stained by silver nitrate. Synthetic telomerase oligonucleotides generated the appropriate 6 bp ladder PCR products. The ability of telomerase in MCF-7/antisense hTR cells to synthesize larger PCR products was obviously inhibited (first lane on the left), compared to the two control cell lines (MCF-7/control, and MCF-7/parental). The first lane on the right is the molecular weight marker utilizing pGEM-7Zf (+) plasmid with *Hae* digestion. **(b)** The telomere length was measured in cells after 15PD (upper panel) and 30PD (lower panel) in MCF-7/parental (left), MCF-7/control (center), and MCF-7/anti-hTR (right). DNAs were digested with restriction enzyme *EcoR*I, separated by electrophoresis on a 0.8% agarose gel and hybridized to the human telomeric probe γ-^{32}P-$(TTAGGG)_4$

Table 1 Antisense hTR significantly suppressed colony formation and cell cycle of MCF-7 breast cancer cells

	% Colonies in soft agar[a]		*% Cells in cell cycle (PD30)*[b]	
	PD20	*PD30*	*G1 phase*	*S phase*
MCF-7/parental	67.8±8.2	64.8±15.1	55.5±5.2	31.5±7.5
MCF-7/control	71.0±4.1	70.3±5.1	56.4±3.3	28.8±2.5
MCF-7/anti-hTR	40.7±1.8[c]	33.8±2.5[c]	68.7±0.3[c]	16.1±3.3[c]

[a]Colony formation in soft agar by MCF-7 cells expressing antisense hTR was compared with controls. Percentage of colonies was calculated as the per cent cells to form clones in soft agar when 500 cells were plated per 35 mm dishes. Values are mean±s.d. of four independent dishes.[b]Cell cycle was analysed in MCF-7/parental, MCF-7/control and MCF-7/anti-hTR cells at PD30. Values are mean±s.d. of four independent experiments.[c]Indicates the statistically significant difference by student's *t*-test ($P<0.05$)

MCF-7/anti-hTR cells were also observed at PD20 and PD30, when compared with controls (data not shown). Expression of antisense hTR by MCF-7 cells inhibited cell proliferation when examined at PD15 and PD25. As shown in Figure 5a, at the early passage of PD5, no significant difference in the proliferation rate was observed among the three cell lines: MCF-7/parental, MCF-7/control, and MCF-7/anti-hTR. At PD15, MCF-7/anti-hTR cells showed moderate but significant slow-down of proliferation from 36 to 60 h after cultures were initiated (Figure 5b). At PD25, the MCF-7/anti-hTR cells showed stronger suppression of cell proliferation from 36 to 60 h after cultures were initiated, when compared with controls (Figure 5c). The initial slower growth rate of MCF-7/anti-hTR cells might be partly because of the cell cycle arrest at G1 phase (Table 1), when the cell cycle analysis was carried out at 48–60 h after cell initiation. However, at both PD15 and PD25,

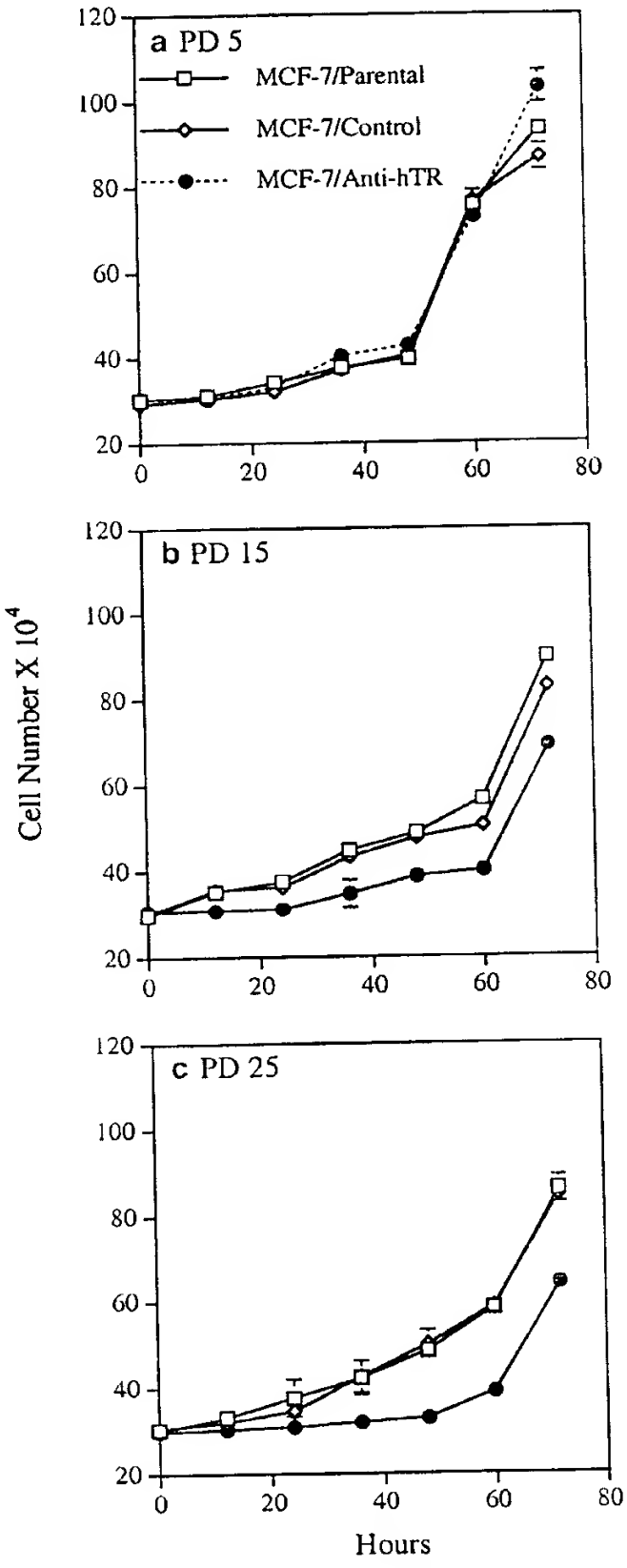
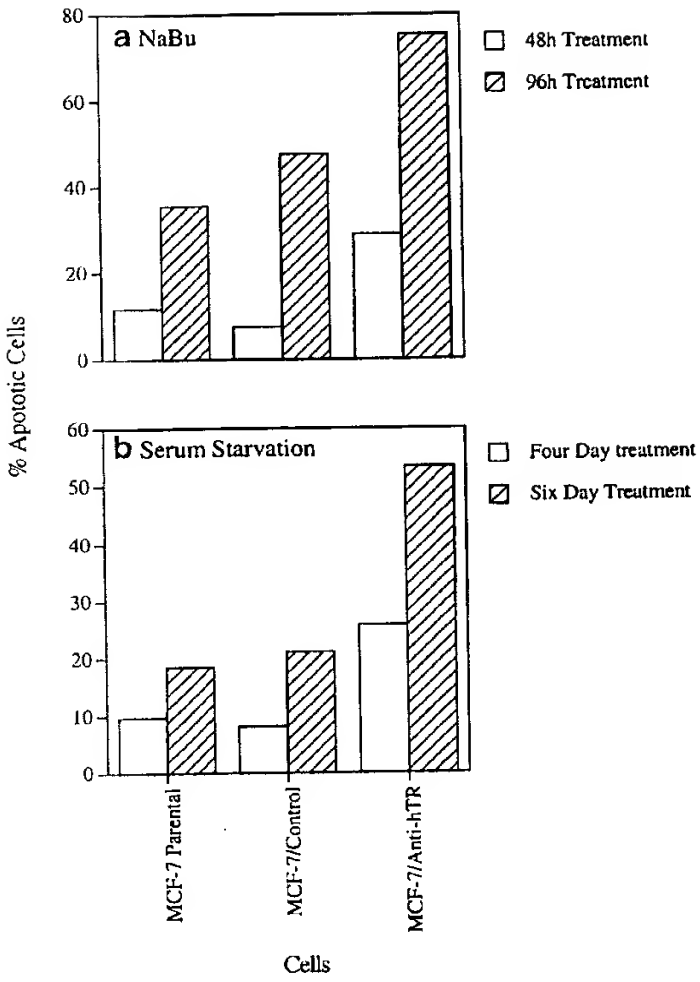


Figure 5 The growth characteristics of MCF-7 cells expressing antisense hTR. The cell proliferation rates at PD5 (a), PD15 (b), or PD25 (c) were determined by counting cell numbers at the indicating time points. Cells (3×10^4) were plated in each 35 mm tissue culture dishes. and six replicates were used for each time point. The data were presented as the mean calculated from the six replicates

all cell lines reached similar growth rates 60 h after cultures were initiated, when the cells entered the log growth phase. The exact mechanism of this phenomenon is not very clear, and it will be interesting to see the difference in cellular adhesion and growth factor dependence of MCF-7/anti-hTR cells when cells enter



Figures 6 Quantification of cell apoptosis in MCF-7 cells expressing antisense hTR treated with NaBu and serum starvation by flow cytometry. Cells were treated with NaBu (a) or serum starvation (b) for the indicated time, and harvested by trypsinization. The cells were fixed, permeablized, digested with RNase, and stained with PI. The percentage cells in sub-G1 phase was gated and calculated as the apoptotic cells

stationary or log growth phase. The significant cell cycle arrest was observed in MCF-7/anti-hTR cells at PD30, when the increased cell number was in G1 phase and decreased cell number was in the S phase (Table 1). Before PD20, there was no significant difference in cell cycle distribution, but increased spontaneous cell death was observed in MCF-7/anti-hTR cells (data not shown). Morphologically, MCF-7 cells expressing antisense hTR exhibited the spindle shapes with granules in cytoplasm and less mitosis, in contrast with that of MCF-7/parental and MCF-7/control cells, which appeared as the classical cuboidal or polygonal adenocarcinoma morphology with larger nucleus, clear cytoplasm, and active mitosis (data not shown).

Antisense hTR enhanced MCF-7 cell apoptosis induced by NaBu or serum starvation

Inhibition of telomerase activity is known to limit cell lifespan by trigging cell senescence or apoptosis. To determine if vAd–AAV-antisense hTR worked additively or synergistically with agents known to induce cell death in MCF-7 cells, we treated MCF-7/anti-hTR cells with 5 mM sodium butyrate (NaBu, Figures 6a and 7a), or serum starvation (Figures 6b and 7b). NaBu is an

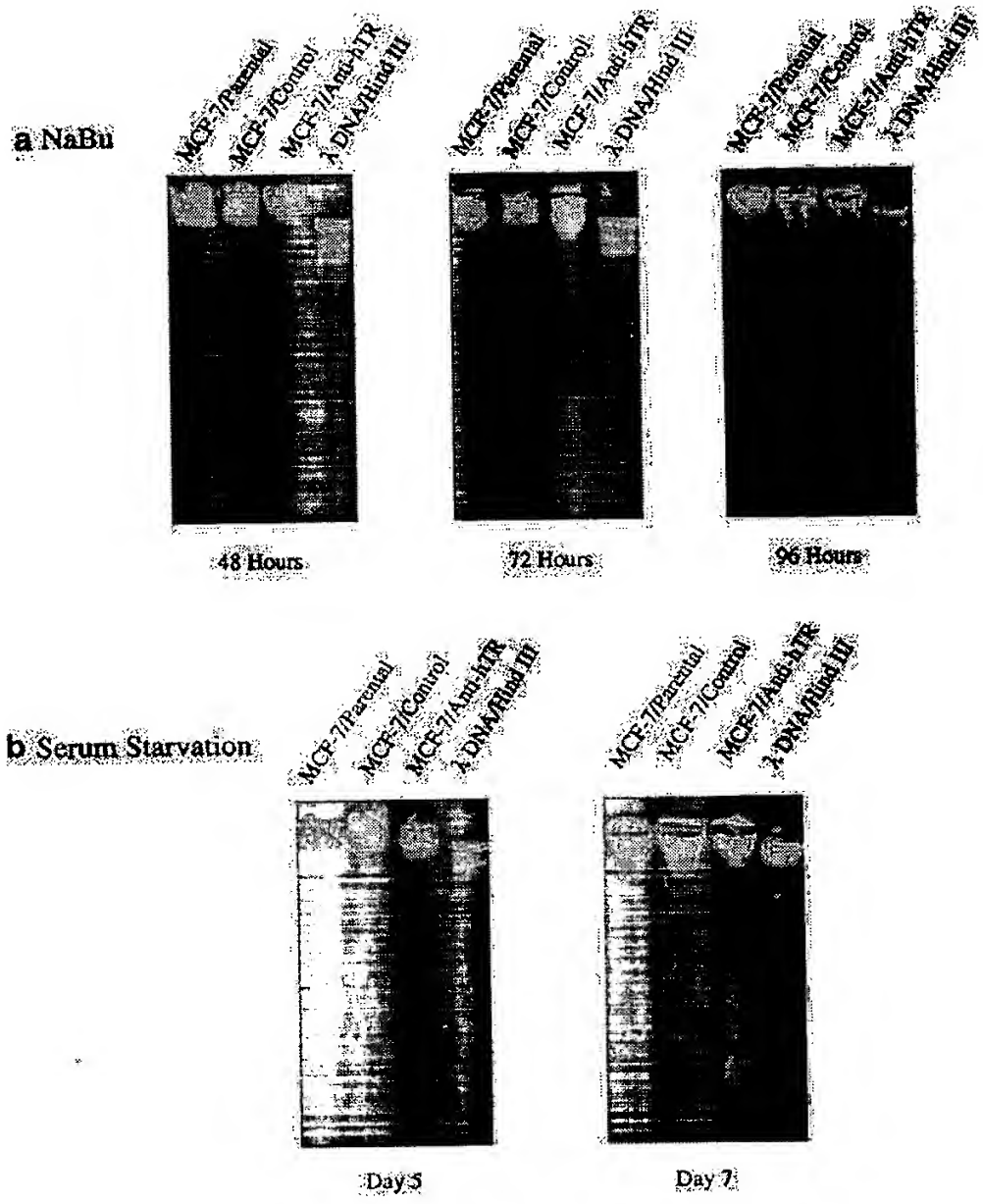


Figure 7 Expression of antisense hTR-enhanced cell apoptosis induced by NaBu and serum starvation. The cultured cells were treated with NaBu (**a**) or adapted into serum-free culture medium (**b**) for the indicated time. After DNA extraction and digestion with RNase, 10 μg of each DNA was electrophoresed on a 1% argarose gel containing ethidium bromide. Gels were visualized under the UV light and photographed. From left, MCF-7/parental cells. MCF-7/control cells, MCF-7/anti-hTR, and DNA ladder standard (λ DNA with *Hind*III digestion)

apoptosis-inducing agent that inhibits histone deacetylation, leading to altered chromatin structure and disrupted DNA replication (Guilbaud *et al*., 1990; Yamamoto *et al*., 1996). Using flow cytometry, quantitative cell apoptosis was measured as the fraction of the fragmented DNA at the sub-G1. In the regular culture medium, MCF-7 cells show no or minimal spontaneous cell apoptosis (<5%, Wang *et al*., 1997b; Wang and Tong, 1999). After cells were treated with NaBu for 48 h, a significant increase of cell apoptosis was observed in MCF-7/anti-hTR cells (29%), in contrast to the slightly increased cell apoptosis (7–11%) in MCF-7/parental and MCF-7/control cells (Figure 6a). After 96 h of NaBu treatment, 75% of cell apoptosis was observed in MCF-7/anti-hTR cells, compared with 35–47% apoptosis in control cells (Figure 7a). The typical laddering pattern of DNA fragmentation was observed in the apoptotic MCF-7/anti-hTR cells after 72–96 h of NaBu treatment, compared with no or less DNA fragmentations of the control cells (Figure 7a). MCF-7 parental and infected cells were also adapted to the serum-free condition to test their serum-independent cell growth (Figures 6b and 7b). MCF-7/anti-hTR showed significantly higher percentage of cell apoptosis after serum starvation for 4 days (26%) or 6 days (53%), when compared with MCF-7/parental cells at day 4 (9.7%), or at day 6 (18.5%), or MCF-7/control cells at day 4 (8.2%), or at day 6 (21.1%, Figure 6b). Significant increased DNA fragmentation was confirmed by gel electrophoresis showing DNA laddering in MCF-7/anti-hTR cells 7 days after serum starvation (Figure 7b). Taken together, the expression of antisense hTR in MCF-7 cells promoted cell apoptosis in combination with apoptosis-inducing agents or serum starvation.

Discussion

We successfully delivered antisense hTR into MCF-7 breast cancer cells through a vAd–AAV hybrid virus.

The hybrid vector enabled the cDNA encoding the antisense hTR to integrate into MCF-7 host genome and stably express antisense hTR. The cells expressing antisense hTR showed suppressed telomerase activity and shortened telomeres. Inhibition of malignant phenotypes in MCF-7 cells included decreased soft agar growth, slowed cell proliferation rate, and blocked cell cycle in G1 phase. Antisense hTR expression also increased the apoptosis index when combined with cell death-inducing agents. Our study confirmed the hypothesis developed that telomerase/telomere maintenance is a key regulatory mechanism determining the fate of cancer cells.

We observed phenotypic changes of cancer cells, such as reduced cell growth at PD15 and decreased colony numbers and sizes at PD20 (Figure 5 and Table 1), before significant telomere shortening at PD30 (Figure 4b). This suggests that antisense hTR-expression-mediated cellular phenotypic alternation in MCF-7 cells may not require a complete inhibition of telomerase activity and significantly uniformed shortening of telomeres. These results also suggest that the experimental measurements of telomerase activity and telomere length may not be sensitive enough to detect early alterations occurring within telomere regions from a given cell population. Consistently, a recent study showed that expression of a mutant hTR inhibited human cancer cell proliferation and enhanced cell apoptosis, without affecting the endogenous wild-type telomerase RNA, endogenous telomerase activity, and telomere length (Kim *et al*., 2001). Based on this observation, it was proposed that uncapping of only one or a few telomeres per cell induced by the mutated hTR-containing telomerase could trigger a DNA damage response. Our experimental observation is consistent with these results in that the antisense hTR-induced suppression of malignant phenotypes (PD15–PD20) occurred prior to the detectable suppressed telomerase activity and shortened telomeres (PD30). In addition, the end structure of telomeres, such as loops or single-strand overhangs, may be important triggers of cells entering senescence or apoptosis (Karlseder *et al*., 2002). Antisense hTR expression in MCF-7 cells may also interrupt the end structure of telomeres, which may lead to the suppression of malignant phenotypes prior to the observed decreased telomerase activity and shortened telomeres. It is important to further investigate the mechanisms of the mutated/antisense hTR-mediated tumor suppression, which may be independent from the telomerase activity or telomere length.

In the present study, we observed that antisense hTR induced both spontaneous apoptosis (dominated before PD20) and senescence (blockage of cell cycle at PD30) in MCF-7 cells, resulting in reduced colony numbers and sizes in soft agar, and slower cell proliferation rates (Figure 5 and Table 1). It has been suggested that induction of cell senescence or apoptosis by affecting telomerase activity and shortening telomere length may depend upon functioning p53, pRb, p16INK4a, or other related molecules controlling cell proliferation, cell cycle, and apoptosis. Karlseder *et al*. (1999) showed that inhibition of telomeric-repeat binding factor 2 (TRF2) by dominate-negative TRF2 induced spontaneous apoptosis in several cell lines, including MCF-7 cells and HeLa cells (cervical carcinoma). The induction of apoptosis depends on wild-type p53 (Karlseder *et al*., 1999). MCF-7 cells are p53 wild type and p16INK4a defective, which might be the important cellular factors affecting antisense hTR-induced spontaneous apoptosis and incomplete senescence. The effects on cell apoptosis and senescence through interaction of antisense hTR with p53 and p16INK4a are under the investigation. It is also interesting to notice that antisense hTR suppressed cell growth more significantly in the stationary phase of cell growth (36–60 h after cell culture initiation), but less significantly at the cell in log growth phase (60 h after cell culture initiation), which happened at both PD15 and PD25 (Figure 5). The mechanism of this phenomenon is not clear, but it suggests that antisense hTR may alter other cellular components affecting cell growth, senescence or apoptosis, such as adhesion molecules, growth factors, or transcription factors.

Although telomerase activity requires coexpression of hTR and hTERT components, hTERT expression is correlated more closely with telomerase activity in cancer cells (reviewed by Elenitoba-Johnson, 2001; Hahn, 2001; Poole *et al*., 2001; Kim *et al*., 2002). Such observations have led to selective anticancer strategies focusing on hTERT (Strahl and Blackburn, 1996; Hahn *et al*., 1999b). For gene therapy, instead of targeting telomerase activity, we pursued targeting of hTR using the hybrid vector. We showed that this strategy effectively suppressed the malignant phenotype of MCF-7 breast cancer cells. Our results are consistent with several previous studies aiming at suppressing telomerase activity by manipulating hTR. Feng *et al*. (1995) originally characterized hTR and revealed that germline tissues and tumor cell lines expressed higher levels of hTR than normal somatic cells and tissues. They demonstrated that transfecting HeLa cells with an antisense hTR resulted in progressive telomere loss and cell death after 23–26 doubling times. Kondo *et al*. (1998) introduced antisense hTR into human glioblastoma U251-MG cells, and showed that antisense hTR either induced cell apoptosis through the mechanism related to increased expression of interleukin-1b-converting enzyme (ICE), or induced cell differentiation by increasing the expression of cyclin-dependent kinase inhibitors (CDKIs) p21 and p27. Bisoffi *et al*. (1998) expressed antisense hTR in HeLa and A-498 human kidney carcinoma, and showed differentiated giant and apoptotic cells after five population doubling time. Kim *et al*. (2001) showed that a low-level expression of mutant template telomerase RNA inhibits cell proliferation *in vitro* and tumor growth *in vivo* of a human breast cancer cell line, MCF-7, and a prostate cancer cell line, LNCaP.

Owing to the fact that more than 90% of cancer cells retained elevated telomerase activity which has an inverse relation to the cell senescence and apoptosis,

the overexpression of telomerase protein and increased telomerase activity have been used as the useful clinical diagnostic and prognostic markers (reviewed by Hahn, 2001; Hiyama and Hiyama, 2002). It has also been hypothesized that suppression of high levels of telomerase activity and shortened telomere length may be synergistic to the existing anticancer chemotherapy. Our study showed that antisense hTR significantly increased apoptotic cells after NaBu treatment or serum starvation (Figures 6 and 7). The mechanisms of apoptosis induced by NaBu and serum starvation are different. It has been suggested that the mechanism of NaBu-induced cell apoptosis may be through the inhibition of histone deacetylases; and the post-translational modifications of histones and chromatin composition alteration may disrupt DNA replication and affect gene expression (Guilbaud *et al*., 1990; Yamamoto *et al*., 1996). The exact mechanisms of apoptosis induced by serum starvation are not clear, likely because of the deprivation of the growth factors that are critical for the activation of signal transduction pathways to support the cell proliferation and survival, such as c-*myc* and AKT (Helbing *et al*., 1998; Shin *et al*., 2001). Overexpression of antisense hTR in MCF-7 cells enhanced apoptosis induced by NaBu or serum starvation, but with the different kinetics (Figures 6 and 7). Up till now, the importance of telomerase activity or the telomere length in apoptosis induced by different agents is not clear. A recent study has showed that telomere dysfunction, rather than telomerase *per se*, was found to be the principal determinant governing cell chemosensitivity, especially to agents that induced double-stranded DNA breaks, such as doxorubicin (intercalation and double-stranded DNA breaks), but not to cisplatin (covalent DNA adduct formation), etoposide (topoisomerase II inhibition), or 5-FU (antimetabolite), in TERC−/− (telomerase RNA gene) INK4a−/− mice (Lee *et al*., 2001). In their knockout model, the chemosensitivity was muted in cells deficient for p53. Thus, the dependence of a functional p53 in chemotherapeutic agent-induced cell death may suggest that the loss of functional p53 triggers the resistance mechanism to compensate for the crisis induced by chemotherapeutic agents or shortened telomeres.

Since targeting at hTR is an attractive gene therapy strategy, it is critical to develop a highly effective delivering vector. We took advantage of the hybrid vector retaining the characteristics of both Ad and AAV (Figure 1). Adenovirus has been prominently utilized as the vehicle in gene therapy because of its high titer production, high infection efficiency, broad host range, capable to carry large exogenous DNA (up to about 30 kb), and to infect both proliferating cells and nonproliferating cells with high infection efficiency (reviewed by Ali *et al*., 1994; Douglas, 1994; Kochanek, 1999). However, the infected Ad stays in the host cells only transiently, since the virus is unable to integrate its DNA into host genome. To achieve the continuing expression of exogenous DNA in host cells, repetitive infection of Ad is needed. However, repeated administering Ad is not effective *in vivo*, since strong antiAd immunity is elicited and the infected cells are cleared quickly after each infection (Schulick *et al*., 1997). In contrast, AAV is able to integrate its DNA into the host genome at chromosome 19 in human cells through the ITRs. AAV-based vectors have limited capacity to carry only a small fragment of exogenous DNA. Its additional limitation is the relatively low infection efficiency that may be because of the low titer production of recombinant virus (Berns and Linden, 1995). Since the ITRs in AAV retain the integration and replication functions without requiring additional viral gene products, they become attractive genetic elements to improve the functions of high-efficient vector system such as adenoviral vector (Richard *et al*., 1989; Andrew *et al*., 1994; Recchia *et al*., 1999; Philpott *et al*., 2002; Shayakhmetov *et al*., 2002). We successfully recombined the two ITRs from AAV into a replication-deficient adenoviral vector flanking CMV promoter, Neo gene, and SV40 promoter/SV40polyA sequence cassette (Figure 1b). We showed that such hybrid vector retained the characteristics of both Ad and AAV, and the hybrid vector-encoded antisense hTR was able to infect cells at high efficiency (Wang *et al*., 1997a), and to integrate hTR into human genomic DNA in MCF-7 cells (Figure 2). However, more studies have to be accomplished before this gene therapy strategy can be introduced into the clinical trial, such as the transfection efficiency in the different types of cancer cells, and the possible toxicity with antisense hTR and the hybrid viral vector. One concern is about anti-hTR sequence-induced toxicity in the normal cells. It is an established phenomenon that the adult normal cells express no or very low level of the telomerase except the actively renewing cells, such as hematopoetic cells (Hiyama *et al*., 1995; Harle-Bachor and Boukamp, 1996; Hsiao *et al*., 1997; Kyo *et al*., 1997; Tsao *et al*., 1997). It will be important to test whether this anti-hTR therapy against cancer will cause the hematopoietic toxicity if the systemically delivery will be used. The second concern is the genotoxicity because of the hybrid vector. The major part of the hybrid vector is from an Ad vector and should have the characteristics of a typical adenoviral vector. The hybrid vector only retained small LTR fragment from AAV, which help the virus integrate into the human chromosome DNA 19q13.3-qter, supposedly. We have not been able to test the exact integrating site of this hybrid vector either in cancer cells or in the normal cell lines. The long-term effects of the hybrid vector should be tested. However, based on the information of the clinical usages of AAV and adenovirual vector as the vehicle for gene therapy, there has not been very severe toxicity because of these viral vectors.

Our study showed that targeting at telomerase RNA by the hybrid vector combining Ad and AAv is an effective strategy that suppressed the malignant cell phenotype in breast cancer cells. In addition, we showed that such a strategy is able to enhance the chemical reagent and serum starvation-induced apoptosis, which could be utilized for the combination therapy in the breast cancer, hopefully resulting in better outcomes.

Materials and methods

Cell culture

293 is a human primary embryonic kidney cell line immortalized by sheared human adenovirus type 5 (Ad5) DNA. 293 cells are particularly sensitive to human Ad and highly permissive for Ad infection. This cell line is used as the host cell for infection, production, and amplification of the hybrid adenovirus/adeno-associated virus vector (vAd–AAV). MCF-7 is a human breast adenocarcinoma line isolated from the pleural effusion of a 69-year-old Caucasian female. Both cells were originally purchased from ATCC (Manassas, VA, USA), and were kindly provided by Cell Biology Institute at Shanghai, China. Both cell lines were cultured in a Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen).

Construct vAd–AAV hybrid vectors

To generate human Ad and AAV hybrid vector, a shuttle vector pAdE1CMVITREXneo (vAd-AAV, Figure 1b, upper diagram) was constructed in our laboratory by Dr Fu Shan Wang (Wang *et al*., 1997a). The vector backbone was a recombinant using pAdE1CMV and pAAV/SVneo, gifts from Dr Tom Shenk of Princeton University (Ferrari *et al*., 1996; Lu and Shenk, 1996), and contained essential components of both Ad and AAV. The structural components present in the shuttle vector from *EcoR*I at 0 position were: 5′ adenoviral sequences with inverted terminal repeat (ITR, 452 bp, 0–1.26 mu, dashed bar), AAV ITRE (180 bp, two black arrows, isolated from pAAV/SVneo; Srivastava *et al*., 1983), CMV-neo expression cassette (CMV promoter (open arrow), neomycin resistant gene (fine dotted bar)), SV40 promoter with multiple cloning sites, SV40 polyA signal sequence (bold dotted bar), AAV ITRX (180 bp, two black arrows, isolated from pAAV/SV40neo), and 3′ adenoviral sequence from 9.2–16.0 mu containing 3′ ITR (dashed bar, 2.5 kb).

pGRN83 is the plasmid with a cDNA insert for hTR and was kindly provided by professor Kevin Kaster (Department of Biochemistry, University of Pennsylvania, Philadelphia, PA, USA). To construct the hybrid vector containing antisense hTR, as shown in Figure 1a, the cDNA of hTR was isolated from the plasmid pGRN83 by *Mlu*I and *EcoR*V double digestion. The 605 bp *Mlu*I/*EcoR*V fragment was purified and blunt-ended by Klenow. The fragment was then inserted into the Klenow-blunted unique *Not*I site of pAdE1CMVITREX-neo to generate pAdT (vAd-AAV-antisense hTR) by T4 ligase. The successful insertion of the 605 bp cDNA fragment was confirmed by *EcoR*I digestion and the antisense orientation of the insert was confirmed by *Hind*III/*Sal*I digestion (Wang *et al*., 1997a).

Generate recombinant viral particles

To obtain the recombinant hybrid virus encoding antisense hTR, 293 cells were cotransfected with each of the hybrid shuttle vector pAdE1CMVITREXneo or pAdT and plasmid pBHG11, using lipofectamine (Invitrogen) according to protocol provided by the manufacturer. pBHG11 is a plasmid DNA carrying Ad5 sequences with deletions in E1 region (0.5–3.7 mu) and E3 region (77–86.2 mu), which was kindly provided by Dr Fu Shan Wang of the University of Pennsylvania (Li *et al*., 2000). Five days after transfection, the crude cell lysate were harvested, serially diluted, and used to infect 293 cells followed by G418 selection. Cell lysate was harvested from the plates where the cytopathic effect (CPE) was clearly detected (Zhang and Tong, 2001). After two more rounds of selection, live viral particles were amplified and the virus titer was determined in the range of 6.2–7.8 $\times$ 10^8 PFU/ml (plague-forming unit). The structural identity of viral DNA was confirmed by restriction enzyme digestion as shown in Figure 1b (Zhang and Tong, 2001).

Infect MCF-7 cells with vAd–AAV and vAdT–AAV

MCF-7 cells were plated at a density of 5 $\times$ 10^5 cells/ml in T-75 flasks 1 day before, and infected with recombinant hybrid virus for an hour at a multiplicity of infection (MOI) of 10, 20 and 100. Fresh culture medium was added to the infected cell culture for 48 h, and then cells were selected under G418 (400 μg/ml, Promega, Madison, WI, USA). After selection for 3 weeks, resistant colonies were counted, and colonies were picked and analysed for the hybrid virus integration with antisense hTR. In total, 15 colonies from the infected MCF-7 cells with appropriate virus integration were pooled for further experiments. These pooled cells were designated as MCF-7/anti-hTR (infected with vAdT–AAV) and MCF-7/control (infected with vAd–AAV), respectively. All cell lines were maintained in the complete DMEM medium under G418 selection.

Genomic DNA hybridization

Genomic DNA was extracted from cells using a DNA extraction kit (Stratagene, La Jolla, CA, USA). DNA (30 μg) was digested with the restriction enzyme *EcoR*I, extracted once with phenol/chloroform (1 : 1), ethanol precipitated, and resuspended in TE buffer. Digested DNA was quantified by fluorometry and 10 μg DNA was run in a 0.8% agarose gel in 0.5 $\times$ Tris-Borate-EDTA (TBE) buffer at 1 V/cm overnight. The gels were denatured for 30–60 min in 0.5 M NaOH and 1.5 M NaCl, and neutralized for 30–60 min in 1 M Tris-HCl (pH 8.0) and 1.5 M NaCl. Following overnight transferring, the blots were dried at 60°C for 2 h, and hybridized with probes labeled with [α-^{32}P]dCTP (Promega) by priming with random oligonucleotide (Prime-a-Gene Labeling System, Promega). After stringent washing, blots were exposed to Kodak XAR-5 X-ray film at −70°C for 48 h.

Northern blot analysis

Total RNA was extracted with TRIZOL reagent following the manufacturer's protocol (Invitrogen). Total RNA (15 μg) was electrophoresed on a formaldehyde-agarose denature gel (1% agarose gel containing 2.2 mol/l formaldehyde), transferred to a nylon membrane (Hybond-N, Amersham Life Science, Buckinghamshire, England), and hybridized with [α-^{32}P]dCTP-labeled human antisense hTR cDNA and β-actin probes (Prime-a-Gene Labeling system, Promega). The membrane was exposed to a Kodak XAR-5 X-ray film at −80°C.

Telomerase assay (TRAP, telomere repeat amplification protocol)

MCF-7 cells were seeded at 10^5 cells/ml and incubated overnight at 37°C. Cells were washed once in phosphate-buffered saline (PBS) and homogenized in 50 μl of ice-cold lysis buffer (10 mM Tris-HCl (pH7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol). After 30 min incubation on ice, the lysates were centrifuged at 100 000 *g* for 30 min at 4°C, and the supernatant was rapidly frozen and stored at −80°C. The protein was quantified using the BioRad Protein Assay (BioRad, Hercules, CA, USA), and an aliquot of 5 μg/μl of protein was used for

each telomerase assay. The extract was assayed in 50 ml of reaction mixture containing 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween-20, 1 mM EGTA, 50 μM dNTP, 0.1 μg of primer1 (5′-AATCCGTCGAGCAGAGTT-3′), 1 μg of T4 gene 32 protein, and two units of *Taq* DNA polymerase (Promega). After 10 min incubation at room temperature for telomerase-mediated extension of the primer1, 0.1 μg of primer 2 (5′-CCCTTACCCTTACCCTTACCCTTA-3′) was added. The reaction mixture was then subjected to PCR amplification in a thermal cycler with 30 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min. The PCR product was electrophoresed on a 12% polyacrylamide gel in 0.5 × TBE. Gels were stained by 0.1% $AgNO_3$ (Promega).

Telomere length analysis

Blots containing genomic DNA were prepared as described in 'Genomic DNA hybridization' and hybridized to end-labeled telomeric oligonucleotide probe [^{32}P-$(TTAGGG)_4$]. After washing, the blots were autoradiographed on Kodak XAR-5 X-ray film for 12–24 h at room temperature. To calculate the telomere restriction fragment (TRF) length, the gel images were scanned with a densitometer and the data were analysed as described (Harley *et al*., 1990). The mean TRF length was defined as: $\Sigma(OD_i)/\Sigma(OD_i/L_i)$, where OD_i is the densitometer output and L_i is the length of the DNA at position i. This method takes into account the greater intensity of signals from larger fragment. The amount of telomeric DNA was calculated by integrating the volume of each smear in ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

Cell morphology, proliferation assay, and soft agar colony assay

For cell morphology, 3×10^6 cells from PD10 were plated in 60 mm tissue culture dishes for 3 days, and microphotographed under the phase contrast DMIL microscopy (Leica Microsystems Ltd, Germany), with magnification of 100-fold. For cell proliferation, 3×10^4 cells were plated in each 35 mm tissue culture dishes, and six replicate dishes were used for each time point. For every 12 h, cells were trypsinized, and counted using hemocytometer. Data were presented as the mean plus s.d. from the six replicates, and the statistical significance was calculated by student's t-test. In soft agar colony assay, 500 cells were suspended in 0.3% agar in DMEM with 10% FBS, and plated on top of 0.6% agar in 35 mm dishes. Cells were allowed to grow in soft agar in 37°C CO_2 incubator for 3 weeks. The numbers of quadruplicate colonies were counted and calculated, and statistical significance was tested by student's t-test.

DNA flow cytometry

Single-cell suspensions were harvested from monolayer cultures after cells were treated with 0.02% EDTA and 0.25% trypsin (Sigma, St Lois, MO, USA). Harvested cells were fixed and permeablized with ice-cold ethanol for 12 h. After treating the cells with 100 μg/ml RNase A (Sigma) at 37°C for 30 min, DNA content was determined by staining with propidium iodide (PI, Sigma) at 1 μg/ml with 0.1% Triton for 30 min. The fluorescence intensity was measured by collecting 10 000 cells using FACScan flowcytometry system (Becton-Dickinson, San Jose, CA, USA). Cell cycle distributions were analysed using CellFit software (Becton-Dickinson, San Jose, CA, USA).

DNA fragmentation assay

The cultured cells were treated with NaBu (5 mmol/l, Osaka Co, Japan) or adapted into serum-free culture medium for the indicated time points. After washing with PBS, cells were scraped, and cell pellets were collected by low-speed centrifugation. The cell pellets were resuspended in lysis buffer (10 mmol/l Tris-HCl, pH8.0, 0.1 mmol/l EDTA, RNase A, 20 μg/ml, and 0.5% SDS), and incubated at 37°C for 1 h. Then protease K was added to the cell suspension at final concentration of 100 μg/ml and incubated for 3 h at 50°C. DNA was extracted three times with phenol saturated with 100 mmol/l Tris-HCl buffer (pH 7.4), precipitated from the aqueous phase with ammonium acetate and ethanol at –20°C, washed with 75% ethanol, and dissolved in Tris-EDTA (TE) buffer. RNA-free DNA was obtained by 20 μg/ml of RNase digestion for 1 h at 37°C followed by phenol extraction twice. A measure of 10 μg of each DNA was electrophoresed on a 1% argarose gel containing ethidium bromide in TE buffer run at 20V for 14 h at room temperature. Gels were visualized under the UV light and photographed.

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